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# THE MECHANISM OF THE INHIBITION OF HEMOLYSIS

## IV. THE TYPES OF REACTION INVOLVED

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(Received for publication, July 5, 1945)

This paper is concerned particularly with the problem of whether the inhibitory effects of plasma components are due to reactions between the inhibitors and the lysins in the bulk phase of the system, or to reactions which involve, and take place at, the red cell surfaces, or to both. The earlier quantitative descriptions of inhibitory phenomena were developed exclusively from the first of these two points of view (Ponder, 1924, 1925, Ponder and Gordon, 1934), but I recently observed that the inhibition produced by plasma depends on the type of cell used in the hemolytic system (Ponder, 1943), and suggested that the inhibition is the result, in part at least, of reactions which involve the cell surfaces. Other investigations (Lee and Tsai, 1942 *a, b*), however, point to the effect of cholesterol, one of the inhibitory components of plasma, as being the result of a reaction between it and the lysin in the bulk phase of the system. The situation is therefore much more complex than has been supposed, and what is true for one inhibitory component is probably not true for another.

From an experimental standpoint, three types of reaction are distinguishable in a system containing red cells, one lysin, and one inhibitor (or, in some cases, one accelerator). (1) The inhibitor may react irreversibly with the components of the red cell surface, and reactions of this kind can be distinguished by the inhibitory effects persisting after the inhibitor has been allowed to remain in contact with the cells for some time, at the end of which it is washed off (Ponder, 1926). (2) The presence of the inhibitor may determine that the lysin is less concentrated at the red cell surfaces than it would be in the absence of the inhibitor. In this case the inhibitory effect does not persist after the inhibitor is washed away, although the involvement of the red cell surfaces can be established independently. (3) The inhibitor may react with the lysin in the bulk phase of the system to render the latter inert. In this case, red cells added to the lysin-inhibitor mixture merely act as an indicator of the amount of free lysin present, the quantity of which can be shown to vary, in some cases, with the temperature at which lysin and inhibitor are mixed (Lee and Tsai, 1942 *a*).

Other possibilities exist, e.g. the reaction between inhibitor and the components of the cell surface might be completely reversible by the process of

washing, or the inhibitor might react with the lysin in the bulk phase to form a new substance of smaller hemolytic activity than that of the lysin itself. In the meantime, there are no experimental means for recognizing such reactions, although they are possible.

In this paper I shall combine a description of some of the inhibitory, and occasionally acceleratory, effects produced by preparations of (*A*) lecithins, (*B*) cholesterol, and (*C*) proteins, with investigations on the nature of the reactions by means of which the effects are brought about. The descriptions of the phenomena themselves are probably quite incomplete, partly because our knowledge of the fractionation of the plasma components is still in a state of development; even now they could be extended by using a larger number of known fractions than I have used. The main purpose of the paper, however, is to direct attention to the various mechanisms underlying the inhibitory phenomena.

#### A. LECITHINS

Because of the prevailing opinion that most preparations of phospholipids are mixtures of none too well defined composition, I have used three substances as representative of the lecithins, recognizing, at the same time, that the lecithins as they occur in plasma may have properties different from the properties of any of these.

(1) Distearyl lecithin, kindly given to me by Dr. Harry Sobotka, is a synthetic preparation made by Dr. Adolph Grün from choline phosphoric acid and distearine. The fatty acid chains are fully saturated, and the substance is very stable. (2) "Lecithin *ab ovo*" is a Merck preparation of egg lecithin, and I have included it because it has been used in the past in so many investigations in which the properties of "lecithin" were being studied. It is certainly a mixture of lecithins with both saturated and unsaturated fatty acid chains, some of which are oxidized. (3) Soybean lecithin, prepared in the laboratory from material kindly sent me by the American Lecithin Co., probably closely resembles egg lecithin in its general properties, but, being freshly prepared, is relatively free from oxidation products.

These three substances were introduced into hemolytic systems in the form of sols. As in the case of cholesterol sols, a question must arise as to the extent to which physical properties, such as dispersion, influence the inhibitory and acceleratory properties observed. It is almost certain that the phospholipids in these sols in saline are not identical with the phospholipids in plasma, either as regards their physical condition or their chemical combination (see Bloor, 1943). The most that their behavior in hemolytic systems can be expected to provide, accordingly, is an indication of the way in which the plasma lecithins may enter into reactions with lysins and with the cell components.

### *I. Sols of Distearyl Lecithin*

1. *Preparation of Sols*—5 mg. of distearyl lecithin is dissolved in 2 ml. of absolute methyl alcohol with the aid of heat. The entire quantity is then added to 10 ml. of isotonic phosphate buffer<sup>1</sup> at pH 6.4, just brought to the boil. After boiling for about 15 seconds, the sol is allowed to cool slowly. It is translucent at first, but it becomes opaque as it stands, and it ought to be freshly prepared before each experiment. Each milliliter of the sol contains 500  $\gamma$  of distearyl lecithin. The sol does not produce lysis by itself, even after several hours, and in this respect differs markedly from the sols made from the commercial "lecithin *ab ovo*" or from soybean lecithin, to be discussed below.

2. *The Inhibitory Effect of Distearyl Lecithin in Lysin-Inhibitor-Cell Systems*.—Table I gives the data for typical time-dilution curves at 37°C. for (a) a standard system containing various amounts of digitonin, saline sufficient to make the volume up to 1.6 ml., and 0.4 ml. of red cell suspension added subsequently, and (b) varying quantities of digitonin, 0.8 ml. of the lecithin sol (400 $\gamma$ ), and 0.4 ml. of suspension added subsequently. Times for complete hemolysis are in seconds.

In this case the  $R$ -value for the inhibition is about 1.6,<sup>2</sup> but the value obtained varies considerably with different sols, and is sometimes as low as 1.15.

Table II shows the results obtained in similar systems containing saponin and distearyl lecithin sol (400 $\gamma$ ) at 37°C. Times for complete hemolysis are in minutes. The average  $R$ -value is about 1.45.

3. *The Order of Mixing the Components*.—Tables I and II refer to systems in which 0.8 ml. of the lysin in various dilutions is allowed to stand together with the inhibitor for a known time (usually about 15 minutes), and the cells added subsequently. Alternatively, the lecithin sol and the cells can be allowed to stand together at 37°C. for varying lengths of time, and the lysin added to complete the system: this is a cell-inhibitor-lysine system, in contrast to the type of system to which Tables I and II refer; *i.e.*, lysin-inhibitor-cell systems.

<sup>1</sup> Isotonic NaCl-phosphate buffer at pH 7.0 is made by adding 25 ml. of a mixed phosphate buffer (72 ml. M/15 Na<sub>2</sub>HPO<sub>4</sub> and 28 ml. M/15 KH<sub>2</sub>PO<sub>4</sub>) to 75 ml. of 1.2 per cent NaCl. Isotonic buffers at other pH's are made by varying the volumes of the two phosphates in the mixed buffer.

<sup>2</sup> This is an average value, for  $R$  is really a function of  $c_1$  (Ponder, 1943), and the values of  $R$  and even more obviously those of  $\Delta$  depend on the initial concentration of lysin in the system. Sometimes  $R$  is constant over a considerable range, in which case  $c_1$  is linear with  $c_2$  and  $\Delta$  is a constant fraction of  $c_1$ . More frequently  $R$  becomes smaller as  $c_1$  decreases, but in some cases  $R$  may increase as the asymptotic values are approached (see Table XII and footnote 20). All kinds of  $R$ - $c_1$  relations seem to be possible as the investigations are extended to a greater variety of systems. Note: In Ponder, 1943, page 6, line 3, and p. 8, lines 5, 11, and 14,  $c_2$  and  $c_1$  should be read instead of  $R$  and  $c_1$ .

Distearyl lecithin is far more inhibitory in cell-inhibitor-lysin systems than it is in lysin-inhibitor-cell systems; *i.e.*, systems containing digitonin, saponin,

TABLE I

	Stock digitonin, ml.*					
	0.5	0.4	0.3	0.16	0.12	0.10
Standard system.....	4	5	7.0	11	19	50
System containing 400γ lecithin.....	5	6	8.5	40	>300	—

\* In making up systems containing saponin, I prepare a series of dilutions of the lysin (2.5 in 10,000, 2.5, in 20,000, etc.) in 100 ml. bottles, and add 0.8 ml. of these various dilutions to the tubes in which the hemolytic systems are contained. In systems of volume 2 ml. they become dilutions of 1 in 10,000, 1 in 20,000, etc. In working with digitonin, on the other hand, I use only two solutions, a "stock" containing 100 mg. of digitonin per liter (dissolved with the aid of heat), and a stock diluted 1 in 5. Various quantities of these, *e.g.* 0.8 ml., 0.7 ml., etc., are added to the tubes containing the hemolytic systems; saline is then added to bring the volume up to 0.8 ml., and the rest of the hemolytic system is completed, to a total volume of 2 ml., in the usual way. The reason for this difference in technique lies in the tendency of digitonin to be concentrated on glass surfaces, and irregular results are obtained if one attempts to prepare a series of dilutions beforehand and to pipette them individually into the tubes containing the hemolytic systems. It is even important to maintain about the same level in the vessel containing the stock solution to be pipetted, so that exposure to the glass surface is approximately constant.

TABLE II

	Saponin, $c_1$ in γ					
	200	133	100	80	67	50
Standard system.....	0.3	0.7	1.5	3.5	7.0	30
System containing 400γ lecithin.....	0.7	2.2	6.0	15	—	—

TABLE III

	Stock digitonin, ml.*					
	0.5	0.4	0.3	0.16	0.12	0.10
Standard system.....	4	5	7.5	11	19	20
System containing 80γ lecithin†.....	5	6.2	8.3	37	300	—

\* Or γ of digitonin  $\times 10^{-2}$ .

† Time of contact between cells and inhibitor, 15 minutes at 37°C.

or taurocholate as lysins and distearyl lecithin as the inhibitor are catastoichic, the inhibitory effect depending on the order in which the components are brought together (see Ponder, 1934). Table III gives values which are

strictly comparable with those of Table I, with the difference that in the case of Table III the systems are of the cell-inhibitor-lysin type and the concentration of inhibitor *one-fifth* that in the lysin-cell-inhibitor systems of Table I. The inhibitory effect of the distearyl lecithin is thus increased *fivefold* by its remaining in contact with the cells for 15 minutes at 37°C.

The same result can be obtained in another way, which can be illustrated by an experiment involving hemolytic systems containing saponin. Three tubes are prepared, the first containing 0.8 ml. of 1 in 15,000 saponin plus 0.8 ml. of phosphate buffer at pH 6.8, the second containing 0.8 ml. of 1 in 15,000 saponin plus 0.8 ml. of lecithin sol in the phosphate buffer, and the third containing 0.8 ml. of lecithin sol in phosphate buffer plus 0.4 ml. of red cell suspension. The three tubes are allowed to stand in the water bath at 37°C. for a known length of time, say 15 minutes (the "time of contact" between cells and inhibitor). At the end of this time 0.4 ml. of cell suspension is added to the first and second tubes, and 0.8 ml. of 1 in 15,000 saponin (at 37°C.) to the third tube. This completes the three hemolytic systems, and the time for complete hemolysis is measured for each. In a typical experiment they were found to be

Lysin + saline + cells	16 seconds.
Lysin + inhibitor + cells	35 seconds.
Cells + inhibitor + lysin	7 minutes.

More inhibition is therefore produced by adding the cells to the inhibitor and allowing them to stand together for 15 minutes than is produced when the cells are added to the lysin-inhibitor mixture; *i.e.*, the system is catastoichic. To determine how much inhibition is produced by allowing the cells and the inhibitor to stand together, we plot a standard time-dilution curve for the lysin, and by referring to it find that 35 seconds is the time required for complete lysis by a 1 in 21,000 saponin, whereas 7 minutes is the time required for lysis by 1 in 30,000 saponin. Now expressing the results as *R*-values, we have  $R = 1.4$  for the lysin-inhibitor-cell system, and  $R = 2.0$  for the cell-inhibitor-lysin system; *i.e.*, allowing the cells and inhibitor to stand together for 15 minutes at 37°C. increases the inhibitory effect  $(2.0 - 1.0)/(1.4 - 1.0)$  times, or 2.5 times, in this experiment.

Similar experiments carried out with sodium taurocholate in phosphate buffer as the lysin, and distearyl lecithin in the same buffer as the inhibitor, show that the inhibitory effect is increased to about the same extent (2 to 5 times) by allowing the cells and the inhibitor to stand together for 15 minutes at 37°C. These effects point to the distearyl lecithin having a direct effect on the components of the surface of the cells.<sup>3</sup>

<sup>3</sup> There are at least three possibilities which account for the inhibition in cell-inhibitor-lysin systems being greater than that in lysin-inhibitor-cell systems. (1) Any direct reaction between the inhibitor and the cell surface components is favored

4. Effects of Distearyl Lecithin at Red Cell Surfaces.—Direct evidence of an effect of distearyl lecithin on the red cell surfaces is provided by two types of observation.

(a) To 5 ml. of red cell suspension is added 5 ml. of a distearyl lecithin sol in phosphate buffer (system B), and to the same amount of suspension in another tube is added 5 ml. of the buffer alone (system A). The two mixtures are allowed to stand for 15 to 30 minutes at 37°C.; they are then centrifuged, the supernatant fluids removed, and 5 ml. of buffer added to each to make two suspensions B and A. Using digitonin or saponin as lysins, time-dilution curves are plotted for both suspensions at 37°C., and the inhibition produced in system B is expressed as an  $R$ -value by referring to the standard time-dilution curve for suspension A. A series of six experiments of this type gave  $R$ -values varying from 1.45 to 1.15, with an average of 1.32. This is less than the inhibition in lysin-inhibitor systems (section 2, above), and considerably less than that in cell-inhibitor-lysin systems (section 3, above), but is nevertheless sufficiently great to show that distearyl lecithin produces some of its inhibitory effects at the cell surfaces themselves.

(b) The clearest evidence of a reaction between distearyl lecithin and the components of the red cell lies in the disk-sphere transformation which takes place when the sol is added to a suspension of washed red cells. If 1 ml. of a sol containing 400 $\gamma$  of distearyl lecithin per ml. is added to 1 ml. of a standard suspension of washed human red cells, the disks become crenated spheres within 5 minutes, and perfect spheres within 10 minutes, at 20°C. The transformation is effected with about the same rapidity by a sol of "lecithin *ab ovo*" containing only 50 $\gamma$  of lecithin, and the difference in activity between

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in the cell-inhibitor system, both because the concentration of inhibitor is greater and because there is more time for the reaction to take place. Evidence of such a direct action is provided in section 4, above. (2) A concentration of the inhibitor at the cell surfaces would provide a region of increased concentration, and this would be a region in which proportionately more lysin might be rendered inert than in the bulk of a homogeneous mixture, since  $R$ , or  $\Delta$ , is a function of the concentration of inhibitor reacting with the lysin. (3) The presence of the inhibitor in the system, whether concentrated at the cell surfaces or not, could so modify the conditions at the cell-fluid interface as to reduce the tendency of the subsequently added lysin to be concentrated there. Except for the experiments described in section 4, above, there is no way in the meantime of determining the extent to which these different processes influence the overall effect. It may be remarked that in the case of possibility (1) the "time of contact" between cells and inhibitor would be expected to be quite important in determining the inhibitory effect, whereas the conditions for possibilities (2) and (3) would probably be established rapidly. It is found experimentally that the inhibition does not usually vary greatly with the time of contact, provided that the latter is longer than half a minute or so.

the two kinds of lecithin may be related to the fact that sols of "lecithin *ab ovo*" are hemolytic, whereas sols of distearyl lecithin are not.<sup>4</sup>

5. *Temperature Effects.*—In the case of mixtures of cholesterol sols and lysins, the inhibitory effect, measured at 37°C., is increased by allowing the inhibitor and the lysin to react together at temperatures from 50° to 70°C. (Lee and Tsai, 1942a). A temperature effect can also be demonstrated in the case of mixtures of distearyl lecithin sols and saponin or digitonin, but it is in the opposite direction. Using 0.16 ml. of stock digitonin as the lysin and 0.8 ml. of a distearyl lecithin sol as the inhibitor, *R*-values of 1.70, 1.60, 1.51, 1.38, and 1.29 were obtained when the lysin and the inhibitor were allowed to react together for 3 minutes at 37°, 50°, 60°, 70°, and 80°C. respectively, the inhibitory effects being measured immediately thereafter at 37°C. If the values are plotted, *R* will be found to be virtually linear with temperature, and the observations provide evidence of a reaction between the lysin and the inhibitor in the bulk phase of the system (a Δ-reaction).<sup>5</sup>

When the distearyl lecithin is heated to 80°C. and then allowed to cool, a sharp increase in its opacity takes place between 70° and 60°C. The optical density values at 4200 Å. are: 80°C., 0.80, 70°C., 0.85, 65°C., 1.20, 63°C., 1.50, 60°C., 1.65, 50°C., 1.75, and 37°C., 1.95. These density changes presumably correspond to changes in micellar dispersion, and it is curious that what probably corresponds to an increase in dispersion (at the higher temperatures) should correspond to a decrease in inhibitory power. The situation is apparently quite unlike that described for cholesterol sols.

## II. Sols of "Lecithin ab Ovo"

1. *Preparation of Sols*—1 gm. of "lecithin *ab ovo*" (Merck) is dissolved in 100 ml. of ethyl alcohol. To 100 ml. of almost boiling isotonic phosphate buffer at pH 6.9, 1 ml. of the alcoholic solution is added dropwise, and the sol is brought to the boil for about half a minute. It is then allowed to cool, and is remarkably stable if kept in the refrigerator at 4°C. Each milliliter contains 100 γ of "lecithin." The sol has hemolytic properties, 0.8 ml. producing just perceptible lysis of 0.4 ml. of a standard suspension of human red cells in 3 minutes at 37°C.

2. *The Effect of "Lecithin" in Lysin—"Lecithin"-Cell Systems.*—The differences between the effects of lecithin *ab ovo* and those of distearyl lecithin seem to be due to the fact that the former substance has lytic properties, and that it probably consists of a mixture of substances which are inhibitory (like distearyl lecithin) and substances which are lytic (like lysolecithin).

<sup>4</sup> The statement that distearyl lecithin does not produce shape transformations (Ponder, 1942, footnote) was made before it was known that stable sols of the substance could be made.

<sup>5</sup> Lee and Tsai (1942 a) did not observe this temperature effect with the lecithin which they used (their Table III and conclusion 3).

In systems containing saponin, the effect of the "lecithin" is that of acceleration between pH 5.0 and 7.0. Table IV, which resembles Tables I, II, and III in its arrangement, shows the values of  $t$  in minutes obtained at pH 6.8 and at 37°C.

Since lecithin *ab ovo* is itself hemolytic, the result in Table IV almost certainly represents a case of one lysin enhancing the action of another. There is little doubt but that the accelerating effect is the result of a reaction between the "lecithin" and some of the components of the red cell surface; this reaction, or others allied to it, produces a disk-sphere transformation of the cells, and the shape change itself is eventually followed by hemolysis (Ponder, 1935,

TABLE IV

	Saponin, $c_1$ in $\gamma$					
	200	100	80	67	50	40
Standard system.....	0.25	1.4	4.5	17	—	—
System containing 80 $\gamma$ "lecithin"*. . . . .	0.11	0.55	0.8	2.0	4.5	8.0

\* The consecutive  $R$ -values are 0.71, 0.66, 0.71, 0.63, and 0.55. The lytic action of the "lecithin" itself would tend to lower the  $R$ -values for the longer times.

TABLE V

	Stock digitonin, ml.*					
	0.4	0.2	0.16	0.14	0.12	0.10
Standard system.....	6	11	17	25	35	60
System containing 80 $\gamma$ "lecithin" . . . . .	4	10	25	120	—	—

\* Or  $\gamma$  of digitonin  $\times 10^{-2}$ .

1942). The surface components, already weakened by the action of the lecithin, are all the more readily broken down by the second lysin, saponin.

In systems containing digitonin, the "lecithin" acts as an accelerator or as an inhibitor, the effect depending on the concentration of the digitonin present in the system. Table V shows the results obtained in digitonin—"lecithin"-cell systems at 37°C.; this table may be compared with Tables I, III, and IV. The times are in seconds.

In the systems containing the higher concentrations of lysin (0.4 and 0.2 ml. of stock digitonin), the addition of the "lecithin" results in an acceleration, whereas in systems containing smaller quantities of lysin there is an unmistakable inhibition. This type of result is obtained from pH 5.0 to 7.0. The time-dilution curves for the standard system and for the system containing the "lecithin" accordingly cross one another (Fig. 1), which is a situation hitherto met with only in cases in which effects at the red cell surfaces are involved

(Gordon, 1933). In this case it is likely that the "lecithin" contains two components, a lytic one and an inhibitory one, and that the effect of the former, at least, is exercised directly upon the components of the surface. Since the time-dilution curves cross one another, we can suppose that the acceleration and the inhibition are equal at the point of crossing; when the system contains

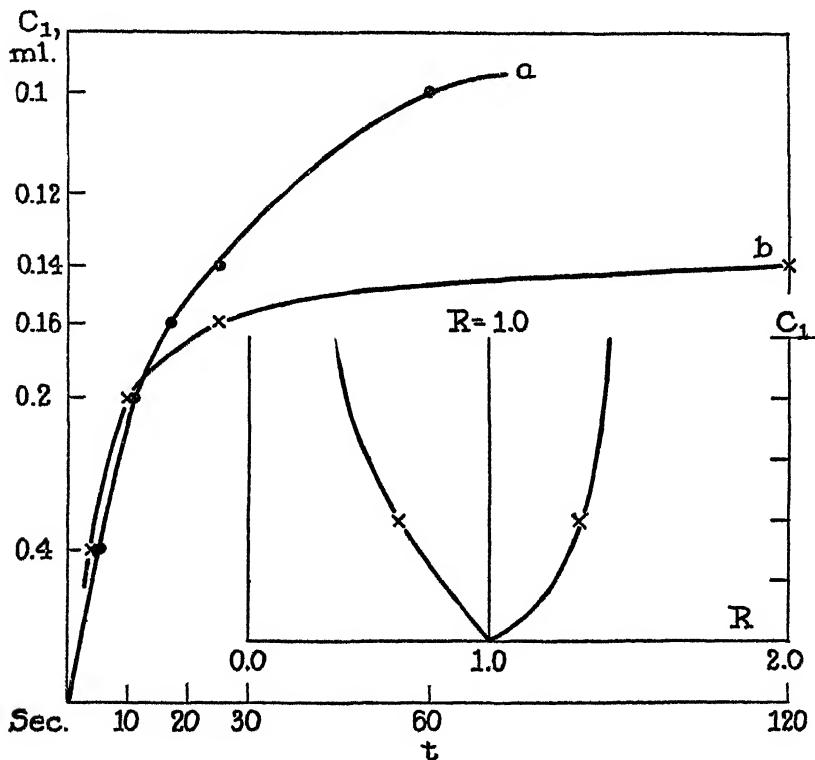


FIG. 1. Ordinate,  $c_1$  plotted on a dilution scale; abscissa, time in seconds. Curve  $a$ , standard system; curve  $b$ , system containing 80  $\gamma$  lecithin *ab ovo*. The inset shows the  $R$ -values of two hypothetical components of the lecithin, one acceleratory and one inhibitory, which would give two time-dilution curves similar to those of the figure. Ordinate,  $c_1$ ; abscissa,  $R$ .

higher concentrations of digitonin, the acceleration exceeds the inhibition, and when the system contains less digitonin, the reverse is the case. The curve-tracing, inset in Fig. 1, shows one familiar type of dependence of  $R$  on lysin concentration which would result in the situation found in experiment.

3. *The Effect of "Lecithin" in Cell-Inhibitor-Lysin Systems.*—In the case of systems containing digitonin in the concentrations for which lecithin *ab ovo* sols act as inhibitors, the inhibition is considerably greater in cell-inhibitor-

lysin systems than it is in lysin-inhibitor-cell systems; *i.e.*, the systems are catastoichic. The results are in general similar to those obtained with distearoyl lecithin as the inhibitor (Table III, compared with Table I), but are more difficult to obtain and to account for because the lecithin *ab ovo* exerts hemolytic effects of its own, as well as inhibiting the action of digitonin. The complicated state of affairs is illustrated by an experiment (Table VI) in which 1 in 15,000 saponin was used as the lysin, 0.8 ml. of the "lecithin" sol (80γ of "lecithin") being allowed to remain in contact with the cells (0.4 ml. of a standard suspension at pH 7.0) for varying lengths of time before the addition of the lysin. The time for complete hemolysis, *t*, is measured from the moment of the addition of the lysin.

TABLE VI

Time of contact at 37°C.	<i>t</i>
min.	min.
0.25	1.9
0.50	1.1
0.75	0.80
1.0	0.65
2.0	0.40
3.0	0.33

*t* for 1 in 15,000 saponin = 0.5. *t* for lysin-inhibitor-cell system = 1.2.

When the time of contact is short (less than about 0.45 minutes) the inhibition is greater in the cell-inhibitor-lysin system than it is in the lysin-inhibitor-cell system, but as the time of contact becomes longer the inhibitory effect diminishes. The simplest explanation is that the inhibition is offset by the effect of the hemolytic component of lecithin *ab ovo*; about 10 per cent hemolysis, indeed, occurs in the cell—"lecithin" system when the time of contact is as long as 3 minutes.

### III. Sols of Soybean Lecithin

1. *Preparation of Sol*—Soybean lecithin is prepared from a mixture of soybean lecithin, cephalin, and lipositol by dissolving the mixture in petrol ether (20 gm. to 200 ml.) and reprecipitating the cephalin and lipositol with 4 volumes of absolute alcohol.<sup>6</sup> After filtration, an additional precipitation with acetone removes fats,

<sup>6</sup> If sols are prepared from the material present in the alcohol-ether mixture (soybean lecithin with some cephalin and lipositol in addition), the freshly prepared sols exhibit a very curious behavior. If the sol is allowed to cool slowly, it is inhibitory for both saponin and digitonin hemolysis. If it is cooled rapidly under the tap, it is much more opaque than the slowly cooled sol, and, while even more inhibitory for saponin hemolysis, is an *accelerator* of digitonin hemolysis. On standing at 4°C. overnight

and the flocculated lecithin, thrown down by centrifuging, is dissolved in a small amount of warm ethyl alcohol. Any traces of cephalin and lipositol which remain precipitate out, leaving the soybean lecithin in alcoholic solution. The quantity contained per milliliter is found gravimetrically. Sols are made by adding 2 ml. of the final solution of the lecithin in alcohol to such a quantity of boiling phosphate buffer at pH 6.8 as will provide a concentration of about 500  $\gamma$  per ml. The sols are faintly opalescent, and are quite hemolytic, 0.8 ml. producing complete lysis of 0.4 ml. of a standard human red cell suspension in 40 minutes at 37°C. The course of the hemolytic process is an unusual one, lysis being very slow to start, but, once started, proceeding rapidly; the result is a positively skew hemolysis curve such as is seen in the case of lysis by the bile salts in certain concentrations.<sup>7</sup> Sols should be used within a few hours of their preparation, as soybean lecithin is unstable in purified form.

TABLE VII

Time of contact at 37°C.	<i>t</i>
min.	min.
0.25	1.9
0.50	1.7
1.0	1.5
2.0	1.2
3.0	1.0

*t* for 1 in 20,000 saponin = 0.7. *t* for lysin-inhibitor-cell system = 1.9-2.1.

*2. Inhibitory Effects of Soybean Lecithin.*—Soybean lecithin is inhibitory for saponin and for digitonin hemolysis in lysin-lecithin-cell systems, 400 $\gamma$  giving an *R*-value of about 1.7 with the former and one of about 1.6 with the latter. In this respect it resembles distearyl lecithin rather than the more hemolytic lecithin *ab ovo*.

In cell-lecithin-lysine systems containing saponin, the inhibition depends, as in the case of cell-inhibitor-lysine systems containing lecithin *ab ovo*, on the length of time during which the cells and the lecithin remain in contact. If the time of contact is very short (10 seconds or so), the inhibition tends to be the same as that produced in the comparable lysin-inhibitor-cell system; I have never observed it to be greater. As the time of contact is increased, the inhibition produced becomes less and less. This is shown in Table VII,

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it loses its opacity and becomes indistinguishable from a slowly cooled sol. The traces of petrol ether and alcohol remaining in the sols seem to have nothing to do with this peculiar behavior, which illustrates some of the difficulties attendant on investigations with this kind of material.

<sup>7</sup> Hemolysis takes so long to begin, and proceeds so quickly once it has started, as to suggest that the soybean lecithin itself is non-lytic, and that it is transformed into a lytic lysolecithin while in contact with the cells.

which refers to an experiment in which 1 in 20,000 saponin was used as the lysin, 0.8 ml. of a soybean lecithin sol containing 200 $\gamma$  of lecithin being allowed to remain in contact with the cells (0.4 ml. of a standard human red cell suspension) for varying lengths of time before the addition of the lysin. The time for complete hemolysis,  $t$ , is measured from the moment of addition of the lysin.

This table may be compared with Table VI. Again the simplest explanation for the result is that the inhibition is offset by the effect of a hemolytic component of the soybean lecithin.

Like lecithin *ab ovo* and distearyl lecithin, soybean lecithin converts washed red cells from disks to spheres without change in volume. Its activity in bringing about this shape transformation is approximately the same as that of lecithin *ab ovo*, a sol containing 100 $\gamma$ /ml. turning the red cells of an equal volume of a standard human cell suspension into spheres in about a minute. The shape change is followed, much more slowly, by hemolysis. It is worth remarking that neither soybean lecithin nor distearyl lecithin contains cephalin, but that both bring about the disk-sphere transformation.

These observations on the lecithins may be summarized by saying that (1) distearyl lecithin is an inhibitor of saponin and digitonin hemolysis, part of the effect being the result of a reaction with the components of the cell surface and part being the result of a reaction with the lysin in the bulk phase of the system, (2) that lecithin *ab ovo* is an accelerator of saponin hemolysis and either an accelerator or an inhibitor of digitonin hemolysis, (3) that soybean lecithin is an inhibitor of both saponin and digitonin hemolysis in lysin-inhibitor-cell systems, but (4) that both soybean lecithin and lecithin *ab ovo* contain a hemolytic (or acceleratory) component.

#### B. CHOLESTEROL

Lee and Tsai (1942 *a*) have shown that if either saponin or digitonin is mixed with cholesterol sols at various temperatures for various times, the mixture being finally cooled to 20°C. and red cells added, the inhibiting effect of the cholesterol depends on the temperature of mixing and the time during which the inhibitor and the lysin have remained in contact. This observation, which can be confirmed in its more general aspects,<sup>8</sup> constitutes very strong

<sup>8</sup> Lee and Tsai did not observe that the lytic activity of saponin is reduced by heating to 60–80°C. In the case of a 1 in 15,000 saponin (133 $\gamma$ /2ml.), the lytic activity is reduced to about 85 per cent of the original value by heating to 75°C. for 30 minutes, and heating to 75°C. for 48 hours reduces the activity to about 45 per cent of the initial value. This effect of temperature on the activity of the lysin is far too great to be ignored in the analysis of the inhibitory effect of cholesterol sols. In a system containing 133  $\gamma$  of saponin at pH 6.5, for example, 4  $\gamma$  of cholesterol inactivates 45  $\gamma$  of lysin after 24 hours at 75°C. and 17  $\gamma$  of lysin after 24 hours at 20°C.; if the effect

evidence for a reaction between lysin and inhibitor in the bulk phase of the system, but the quantity of lysin apparently rendered inert depends also on the type of red cell used (Ponder, 1943), and this shows that a simple irreversible combination of lysin and inhibitor in the bulk phase does not constitute the entire explanation. Experiments similar to those of section A, 4(a) (above) do not provide any evidence of an irreversible reaction between cholesterol sols and the components of the red cell surface, yet a change in the nature of the cell is able to bring about a change in the extent of the inhibition observed under conditions which are otherwise constant. This is shown by the following type of experiment.

Various amounts of a cholesterol sol diluted 1 in 10 (Lee and Tsai's method of preparation) are allowed to react with 0.8 ml. of lysin at 37°C. for 24 hours, the volume

of temperature on the lysin is not taken into account, however, the quantity inactivated at 75°C. appears to be 75 γ instead of 45 γ. Although it is clear that much greater inactivation occurs at higher temperatures than at lower, Lee and Tsai's description of the effects cannot be confirmed in its details. The effect seems to be one on the equilibrium, as well as on the velocity, of the cholesterol-lysin reaction, and its analysis is greatly complicated by the effect of temperature on the activity of the lysin itself.

In the case of digitonin, the lytic activity is not changed by heating, and so an attempt can be made to analyze the effects of temperature on the inhibitory effect. Table VIIIA shows the results of an experiment in which various quantities of digitonin were allowed to remain in contact with 2.5 γ of cholesterol for 24 hours at 20° and at 80°C. The inhibition produced was determined at 37°C. at the end of that time.

TABLE VIIIA

$c_1$	20°C.		80°C.	
	R	Δ	R	Δ
40.0	1.35	10.4	1.51	13.5
26.7	1.50	8.9	1.70	11.0
23.4	1.60	8.8	1.87	10.9
20.0	1.78	8.8	2.0	10.0
16.7	2.1	8.7	2.5	10.0

It will be observed that R increases, and Δ decreases, as  $c_1$  decreases, and that although the effect of the 60°C. increase in temperature is an increase in the inhibition produced by the cholesterol, the increase is far less than Lee and Tsai describe. As the asymptotes of the time-dilution curves are approached, the values of R and of Δ become increasingly uncertain, and this uncertainty is added to by irregularities in the inhibitory effects of cholesterol which are encountered in experiments of this type, in which the lysin and inhibitor remain in contact for very long times. As yet, I have not been able to analyze the temperature effects in any simple way.

of the system being always made up to 1.6 ml. with saline; 0.4 ml. of a standard human or rat red cell suspension is added at the end of that time, and the times for complete hemolysis are determined. Values of  $R$  and of  $\Delta$  are obtained by reference to time-dilution curves for systems containing human and rat cells respectively. Table VIII gives results for 1 in 20,000 saponin (100  $\gamma$ ), with the times for complete hemolysis in minutes.<sup>9</sup>

If  $R$ , or  $\Delta$ , is plotted against  $Q$  for human and for rat cells respectively, the relations will be found to be flat curves<sup>10</sup> which do not by any means coincide with each other. This means that the added red cells do not act simply as an indicator of the amount of lysin left free after the lysin-inhibitor combination in the bulk phase is completed. The results, indeed, are very similar to those which show that the inhibitory effect of plasma on saponin hemolysis depends on the type of red cell added to the system (Ponder, 1943, Table IV), and they can be compared with the results obtained when plasma, diluted 1 in 100, is

TABLE VIII

$Q$ Sol/10 ml.	Human				Rat			
	$t$	$c_2$	$R$	$\Delta$	$t$	$c_2$	$R$	$\Delta$
0.3	—	—	—	—	80	37	<b>2.70</b>	63
0.2	50	48	2.08	52	2.5	42	2.38	58
0.1	2.3	67	1.49	33	0.50	57	1.76	43
0.05	1.1	80	1.25	20	0.25	70	1.43	30

used as an inhibitor with 1 in 20,000 saponin in systems containing human or rat red cells (Table IX).

Again the relations between  $Q$  and  $R$  are flat curves concave to the  $Q$ -axis, and the curve for the system containing rat cells is not at all the same as that for the system containing human cells.

The simplest explanation of the difference in inhibitory power observed when the type of red cell in the system is varied is that the addition of the

<sup>9</sup> In order to ascertain the reproducibility with which inhibitory phenomena such as these can be measured, I made 3 cholesterol sols from solution in ethyl alcohol and 3 sols from solution in methyl alcohol, and measured  $R$  after 24 hours' contact of several different quantities of sol with 133  $\gamma$  of saponin at 37°C. The 6 values of  $R$ , which lay between 1.2 and 2.1 for different quantities of sol, agreed to within  $\pm 0.1$  with each other in the case of any one quantity of sol. There were no consistent differences in inhibitory power between the sols.

<sup>10</sup> There seems to be no doubt that the relation of  $R$  and  $Q$  is really a flat curve rather than the straight line which I described for saponin-plasma systems in 1943. The points which decide the matter in favor of the curve are in bold face type in Tables VIII and IX.

cells brings about a redistribution of the quantities of lysin, free on the one hand and inhibited as the result of a combination with cholesterol or plasma components on the other, and that the way in which the quantities are redistributed depends on the surface properties of the type of red cell added. More specifically, when an inhibitor such as plasma or cholesterol sol is added to a lysin in concentration  $c_1$ , the lysin becomes divided into a quantity  $\Delta$  rendered inert by the inhibitor and a quantity  $c_2$  left free. When red cells are added to the system, lysin tends to become concentrated at their surfaces, so that  $c_1$  is further divided into  $\Delta'$ , a new amount combined with the inhibitor,  $c_2'$ , a new amount left free, and an additional amount concentrated at the cell surfaces. This last quantity seems experimentally to be linear with  $c_1$  and is denoted by  $\xi c_1$ . As the equilibrium between  $c_2$  and  $\Delta$  seems to be at least partially reversible, there arises a competition for lysin between the inhibitor and the red cell surfaces (Ponder and Gordon, 1934, Ponder, 1945 a), and one

TABLE IX

$Q$ , Plasma/100 ml.	Human				Rat			
	$t$	$c_2$	$R$	$\Delta$	$t$	$c_2$	$R$	$\Delta$
0.4	—	—	—	—	2.3	49	<b>2.04</b>	51
0.3	6.0	57	1.75	43	1.1	52	<b>1.91</b>	47
0.2	2.2	69	1.45	31	0.8	55	<b>1.82</b>	45
0.1	1.3	77	1.30	23	0.27	65	<b>1.54</b>	35
0.05	1.0	85	1.18	15	0.22	72	<b>1.38</b>	27

of the principal effects of this competition is to make  $\Delta$ , the amount of lysin rendered inert by the inhibitor, dependent on (a) the number of red cells added, and (b) the kind of red cells added, for both the extent and the nature of the competing red cell surface can influence  $\xi$ .

Tables VIII and IX show that the kind of cell determines the values of  $\Delta$  observed ( $\Delta = c_1 - c_1/R$ ), and it has already been shown that the number of cells added to the hemolytic system determines the values of  $\Delta$  when plasma is the inhibitor and saponin the lysin (Ponder and Gordon, 1934). Table X gives the result of an experiment in which saponin was used as the lysin and 0.1 ml. of cholesterol sol diluted 1 in 10 (5γ of cholesterol) as the inhibitor, and in which 0.4 ml. of a red cell suspension of twice standard strength (2S), standard strength (S), and half standard strength (0.5S) was added after the lysin and the inhibitor had remained in contact for 24 hours at 37°C.

Proceeding in the same way as when the inhibitor is plasma (Ponder and Gordon, 1934), and plotting  $\log \Delta$  against  $\log (c_1 - c_{1\infty})$ , we get three lines parallel to each other (values for  $c_{1\infty}$ , 80γ, 75γ, and 65γ respectively). This is a result similar to that obtained when the inhibitor is plasma, and the same

explanation; *viz.*, that the differences in the observed values of  $\Delta$  are due to redistribution of lysin among the phases of the hemolytic system, appears to be applicable to both inhibitors.<sup>11</sup> By an extension of the same idea, it can be supposed that a change in the *kind* of red cell (but with the same development of surface<sup>12</sup>) operates in the same way as a change in the *number* of red cells of the same kind, both bringing about a change in  $\zeta$  and in the extent to which the cell surfaces can compete for lysin with the inhibitor.

It therefore appears that although lysins such as saponin and digitonin react with cholesterol in mixtures of lysin and cholesterol sol, the extent of the lysin-inhibitor reaction which results in the rendering inert of lysin is finally determined by the affinity of the lysin for the red cell surface components, once the cells are added to complete the hemolytic system. In general, the

TABLE X

Suspension	$c_1$	$t$	$c_2$	$\Delta$
2 S	133	1.7	88	45
	100	17	65	35
S	133	1.5	80	53
	100	8.0	59	41
0.5 S	133	1.6	56	77
	100	6.0	42	58
	80	50	33	47

reactions between lysin and inhibitor which occur in the bulk phase of the hemolytic system cannot be considered apart from the reactions which take place in the neighborhood of the cell surfaces,<sup>13</sup> unless the lysin-inhibitor reaction is known to be wholly irreversible.

<sup>11</sup> If  $\log \Delta$  is linear with  $\log (c_1 - c_{1\infty})$ , it will also be linear with  $\log \zeta (c_1 - c_{1\infty})$ . The slope of the line will be the same, but it will be displaced to the left by  $\log \zeta$ .

<sup>12</sup> A standard suspension of human red cells (area,  $160\mu^2$ ,  $6.(10^6)$  cells per mm.<sup>3</sup>) has approximately the same surface area as a standard suspension of rat red cells (area,  $100\mu^2$ ,  $9.(10^6)$  cells per mm.<sup>3</sup>).

<sup>13</sup> For a description of the different behavior of saponin, digitonin, and the bile salts, see Ponder, 1934, where a distinction is made between "initial absorption," or the condensation of lysin at the red cell surfaces within a short time (10 seconds) after the addition of the cells to the lysin, and "delayed absorption," the more protracted using up of lysin which occurs during, and after, the hemolytic process. The distinction can be made experimentally, but is probably not as sharp as is implied. The "absorption" of lysin at the cell surfaces which has to be considered when one is talking about "competition" is probably that which takes place during the whole of the period of time in which the lysin-inhibitor compound is reversible and can

## C. PROTEINS

Human serum globulin, fraction II + III (a mixture of gamma and beta globulins kindly supplied me by Dr. E. J. Cohn and Dr. H. B. Vickery) is inhibitory for hemolysis by saponin, digitonin, sodium taurocholate, and sodium oleate, the *R*-values ranging from 1.10 to 1.20 for a 0.02 gm. per cent solution at pH 7.1 and at 37°C. (Ponder, 1944). It is important that the globulin solution be freshly prepared.

Attempts to show that serum globulin reacts with the components of the red cell surfaces, producing changes which persist after one washing (*cf.* section A, I, 4(a)), have resulted in failure. The inhibitory effects appear to be due entirely to reactions which occur between the globulins and the lysins in the bulk phase of the system, except in so far as they are modified by  $\zeta$ -reactions (*cf.* section B). Comparing the *R*-value for 0.02 per cent globulin and digitonin, 1.14, with that for diluted plasma with a globulin content of 0.02 per cent, the globulin in plasma can be responsible for only about 10 per cent of the total inhibition, or even less. All calculations of this kind lead to the same general conclusion; *viz.*, that the globulins are responsible for only a small part of the total inhibition produced by plasma.

The effects of serum albumin are very much more complex. When I reported that human serum albumin is an accelerator of saponin hemolysis (Ponder, 1944), the experimental range was apparently insufficiently explored. Actually, human serum albumin (0.1 per cent in saline at pH 7.1) is an accelerator of saponin hemolysis when the lysin is present in low concentration, but an inhibitor when the lysin is present in high concentration. The time-dilution curves for the standard system and the system containing the serum albumin accordingly cross one another, a state of affairs similar to that met with in the case of lecithin *ab ovo* and digitonin (section A, II, 2), except that the position of the two curves is reversed (Table XI).

Table XI shows that there is a small effect of the length of time during which the lysin and inhibitor remain in contact. These systems do not appear to be catastoichic, but the inhibition produced depends on the type of red cell used. In Table XII, which illustrates this, the inhibitor was 0.8 ml. of 0.1 per cent human serum albumin (HA 139).

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give up lysin to augment  $c_2$ , the quantity of lysin which is drawn upon by "initial absorption" and also by "delayed absorption." As yet, the duration and extent of the reversibility are not known: in fact, the whole theory of the redistribution of lysin between the phases of a hemolytic system is only in a very elementary state (*cf.* Ponder, 1945 *a*, in which "competition" is discussed in terms of "affinity" of the cell surface for the lysin. Here the term "affinity" would have to be taken to mean initial plus delayed absorption; *i.e.*, all the ability of the cell to react with lysin, quickly or slowly, throughout the course of the reaction studied).

In systems containing 0.1 per cent human serum albumin and digitonin, I have been able to obtain only the small acceleration already reported (Ponder, 1944) over the entire experimental range ( $80\gamma$  to  $10\gamma$ ). In the case of sodium taurocholate, however, the statement that human serum albumin produces no inhibition must be withdrawn. In systems containing 0.8 ml. of lysin in isotonic NaCl-phosphate buffer at various pH's between 5.8 and 8.5, 0.8 ml. of 0.1 per cent human serum albumin in saline, and 0.4 ml. of standard human red cell suspension, inhibitory effects are observed over the entire experimental

TABLE XI

	Saponin, $c_1$ in $\gamma$					
	100	87	75	62	50	38
Standard system.....	0.20	0.30	0.52	0.90	4.0	>30
System containing 0.1 per cent HA 139,* contact 5 min.....	0.42	0.57	0.80	1.1	2.9	10
System containing 0.1 per cent HA 139, contact 90 min.....	0.50	0.67	0.90	1.2	3.0	10

\* HA 139 is a preparation of human serum albumin kindly sent me by Dr. E. J. Cohn.

TABLE XII

Saponin $\Delta$	Human cells		Rat cells	
	$R$	$\Delta$	$R$	$\Delta$
100	1.46	31	1.11	10
87	1.49	28	1.13	10
75	1.62	29	1.18	12
62	1.94	30	1.39	17

range ( $2000\gamma$  to about  $500\gamma$  of lysin at  $37^\circ\text{C}$ .). The  $R$ -values obtained are shown in Fig. 2. The average  $R$ -value in the range  $\text{pH} = 6.5$  to  $7.7$  is between 1.1 and 1.2, while at higher and lower pH's the values rise to about  $2.0^{14}$ . At  $\text{pH } 7.1$ , 0.1 per cent serum albumin is about as inhibitory as plasma diluted 1 in 500 (see Ponder, 1945 *a*, Table II); *i.e.*, roughly 10 per cent of the inhibitory power of plasma, in this dilution, can be attributed to its contained albumin.

I have not been able to show that these lysin-albumin-cell systems are catastoichic, or that the albumin produces changes at the red cell surfaces which are not reversible with one washing (*cf.* section A, I, 4(*a*)). Independent

<sup>14</sup> At the lower pH's a white cloud appears when the lysin and the serum albumin are mixed. A similar cloud appears under certain conditions when serum is added to sodium taurocholate or glycocholate (Ponder, 1924).

evidence that serum albumin reacts directly with the components of the red cell surface is provided, however, by the effects which it produces on red cell shape and its transformations (see Ponder, 1945 *c*, for the complete literature).

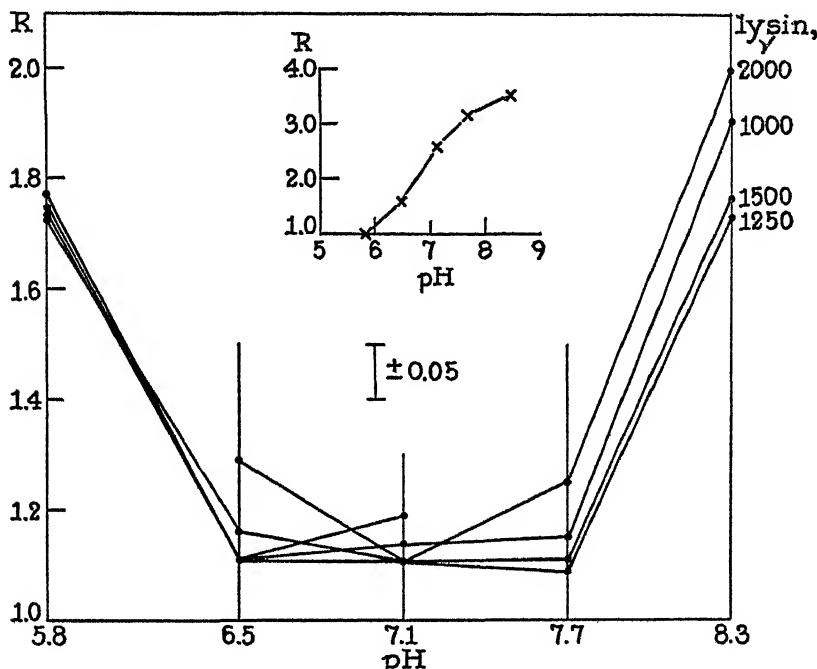


FIG. 2. Inhibition of sodium taurocholate hemolysis ( $c_1 = 2000, 1500, 1250$ , and  $1000 \gamma$ ) by 0.1 per cent human serum albumin (HA. 139) at different pH's. Time of contact, 10 minutes at  $37^\circ\text{C}$ . Ordinate,  $R$ -values; abscissa, pH. Magnitude of allowable experimental error in  $R$ ,  $\pm 0.05$ . Inset shows how the activity of the lysin, expressed as an  $R$ -value with the activity at pH 5.8 put arbitrarily = 1.0, varies with pH.

#### D. OTHER QUANTITATIVE OBSERVATIONS

*1. Catastoichic Inhibition by Plasma.*—Since the inhibition produced by lecithin, one of the components of plasma, is catastoichic in systems containing saponin, digitonin, or taurocholate, one would expect the inhibition produced by plasma to be catastoichic too. A dependence on the order of mixing the components of the hemolytic system, however, has not yet been observed in systems in which the lysin is saponin or digitonin, although it has often been described in systems in which the lysin is one of the bile salts or soaps (Ponder, 1922, Ponder and Gordon, 1934). The explanation for this lies in the fact

that the catastoichic inhibition produced by plasma in saponin and digitonin systems appears only when the plasma is dilute and the inhibition small. This can be shown by measuring the time for complete hemolysis for three types of system: (a) lysin-saline-cell systems, (b) lysin-plasma-cell systems, and (c) cell-plasma-lysin systems, the dilutions of lysin and of plasma being varied to cover an extensive experimental range (Table XIII).

A simple explanation of the catastoichic effects being observed only in systems containing dilute plasma is that the total inhibition produced by plasma is the sum of that resulting from a  $\Delta$ -reaction between plasma components (*e.g.*, cholesterol) with lysin in the bulk phase of the system, and that resulting from effects at the red cell surfaces. The former effects are much greater than the latter, and obscure the smaller effects except when the inhibitor is dilute. A detailed analysis of the situation would be rendered very difficult

TABLE XIII

Saponin 1 in	A	Type of system									
		Plasma 1/100		Plasma 1/200		Plasma 1/400		Plasma 1/800		Plasma 1/1600	
		B	C	B	C	B	C	B	C	B	C
10,000	0.12	3.0	3.0	0.5	0.5	0.22	0.32	0.17	0.23	0.14	0.17
15,000	0.22	—	—	2.3	2.5	1.0	1.3	0.46	0.60	0.33	0.50
20,000	0.83	—	—	13.0	18.0	3.7	4.7	1.6	2.7	1.3	2.0

In column A, times in minutes for lysin-saline-cell systems. In column B, times in minutes for lysin-plasma-cell systems, contact 10 minutes. In column C, times in minutes for cell-plasma-lysin systems, contact 10 minutes.  $t$  for 1 in 30,000, 5.5 minutes.

by the fact that the inhibitory effects, both in the bulk phase and in the neighborhood of the cell surfaces, depend on the times of contact between lysin and inhibitor and between inhibitor and cells (see section 2, below).

2. *Time Effects.*—In their study on the effects of temperature on the inhibition produced by cholesterol, Lee and Tsai showed that the amount of saponin inhibited by cholesterol sols increases with the time during which the lysin and the inhibitor are allowed to react in a lysin-inhibitor system, a final value being reached only after some hours. It is the velocity of this reaction (as well as its equilibrium) which is affected by change in temperature.

In the case of the lecithins, the reaction in a lysin-inhibitor system (the cells being added subsequently) which results in inhibition occurs rapidly, so that it is immaterial whether the lysin and the inhibitor are allowed to react for a long time interval before the addition of the cells or whether the cells are added soon after the lysin and the inhibitor are mixed. When the inhibitor is cholesterol sol or plasma, on the other hand, the time interval is very important in determining the final result, and the course of the reaction between

lysin and cholesterol is not the same as that of the reaction between lysin and plasma. This is illustrated by Fig. 3 A, which shows the results obtained in an experiment in which 25 $\gamma$  of digitonin was allowed to react in a series of

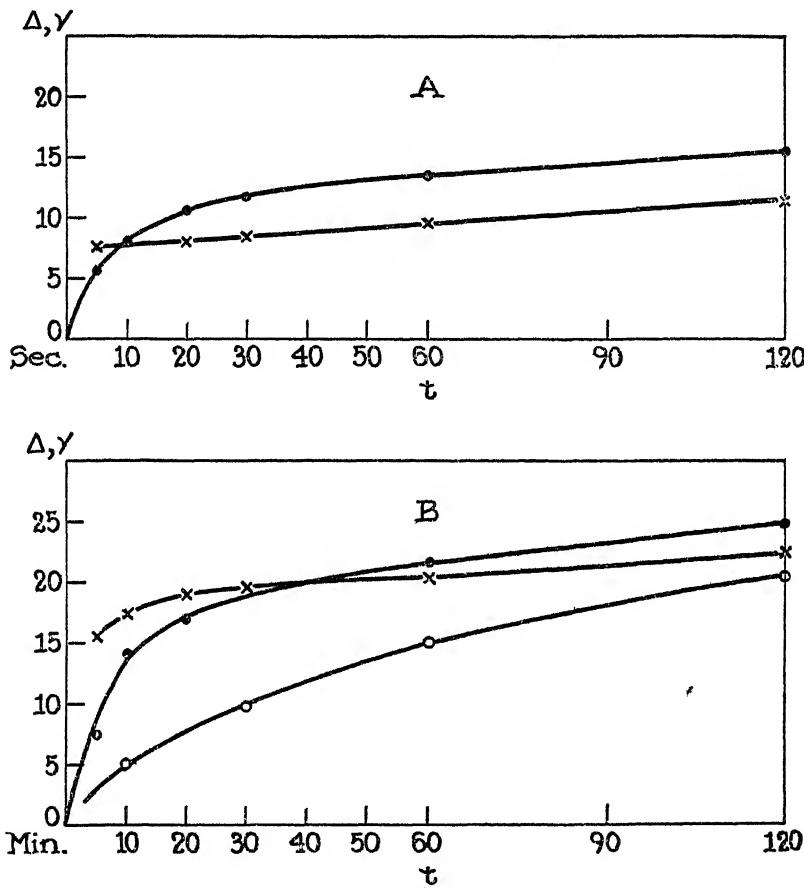


FIG. 3. A, the inhibition of 25  $\gamma$  of digitonin by 2.5  $\gamma$  of cholesterol (solid points) and 0.3 ml. of plasma diluted 1 in 100 (crosses). B, the inhibition of 100  $\gamma$  of saponin by plasma (crosses), 2.5  $\gamma$  of cholesterol (solid points), and 1.25  $\gamma$  of cholesterol (open circles). In both cases,  $\Delta$  is plotted on the ordinate and time on the abscissa.

tubes for 5 minutes, 10 minutes, . . . 120 minutes<sup>15</sup> at 37°C. and pH 7.1 with (a) 0.8 ml. of a cholesterol sol diluted 1 in 160, i.e. with 2.5 $\gamma$  of cholesterol,

<sup>15</sup> The reactions are virtually complete within 120 minutes, the amount of lysin inhibited being the same, within the limits of experimental error, as after 24 hours' contact.

and (b) 0.3 ml. of plasma diluted 1 in 100. The inhibition produced at the end of each period of time was measured in the usual way and the values of  $\Delta$  are plotted against time in Fig. 3 A. Fig. 3 B shows the results in a similar experiment using 100 $\gamma$  of saponin, 2.5 $\gamma$  and 1.25 $\gamma$  of cholesterol, and 0.8 ml. of plasma diluted 1 in 800.

When the inhibitor is plasma, the course of the reaction resulting in the inhibition of the lysin is clearly very different from that observed when the inhibitor is cholesterol sol, irrespective of whether the lysin is saponin or digitonin. When the inhibition at equilibrium (1 to 2 hours contact) is approximately the same, the inhibition produced by plasma is much greater for short periods of contact with either lysin, and the values of  $\Delta$  vary less with time than do the values obtained with cholesterol sol as the inhibitor. If we enquire as to the amount of cholesterol sol which is equal in inhibitory power to a certain amount of plasma, we get a variety of answers (0.3 ml. of plasma

TABLE XIV

Type of system	Time of contact	
	10 min.	60 min.
Lysin + 2 $\gamma$ cholesterol.....	13	19
Lysin + 200 $\gamma$ lecithin.....	13	13
Sum of these.....	26	32
Lysin + 2 $\gamma$ cholesterol + 200 $\gamma$ lecithin.....	27	51

diluted 1 in 100 equal to anything from 5 $\gamma$  to 15 $\gamma$  of cholesterol, in the case of Fig. 3 A) according to the time of contact between the lysin and the inhibitor before the addition of the cells. It can also be shown that a similar variety of answers is obtained when the time of contact is constant, but the initial concentration of lysin is varied (see section 4 below).

3. *Enhancing Effects.*—Lee and Tsai (1942 b) have described a reinforcement of the antihemolytic power of cholesterol by lecithin, their lecithin, although slightly lytic in itself, enhancing the inhibitory effect of a cholesterol sol 3 to 10 times in saponin systems. I have had no difficulty in confirming this fundamental observation. Using four systems, (a) 0.8 ml. 1 in 20,000 saponin (100 $\gamma$ ) plus 0.8 ml. NaCl-phosphate buffer at pH 7.0, (b) 0.8 ml. lysin plus 0.4 ml. distearyl lecithin sol in phosphate buffer plus 0.4 ml. buffer, (c) 0.8 ml. lysin plus 0.4 ml. cholesterol sol diluted 1/100 in buffer (2 $\gamma$  cholesterol) plus 0.4 ml. buffer, and (d) 0.8 ml. lysin plus 0.4 ml. lecithin sol plus 0.4 ml. cholesterol sol, and adding 0.4 ml. of standard red cell suspension to each system after various lengths of time at 37°C., we get, by reference to a standard time-dilution curve for the lysin, the quantities of lysin rendered inert which are shown in Table XIV.

When the time of contact between the inhibitors and the lysin is 60 minutes, the quantity of lysin rendered inert,  $51\gamma$ , is 1.6 times the sum of the quantities of lysin rendered inert by the two inhibitors acting separately, but when the time of contact is much shorter (10 minutes), the enhancement is much less in evidence. Any further description of these complex enhancing effects is beyond the scope of this paper, but it will be clear that the existence of such effects makes it very difficult to say how much of the inhibitory effect of plasma can be attributed to cholesterol, to lecithin, or to other components.

*4. Combining Ratios.*—Even in the case of lysin-inhibitor-cell systems in which the time of contact between lysin and inhibitor is not important, the quantity of lysin inhibited per  $\gamma$  of inhibitor depends on the initial concentration of the lysin, as well as on other factors such as pH, the kind and number of red cells in the system, etc. In the case of distearyl lecithin and digitonin (Table I), each  $\gamma$  of lecithin inhibits  $0.025\gamma$  of lysin when the initial lysin concentration is  $50\gamma/2\text{ml.}$ , and  $0.015\gamma$  when the initial concentration is  $16\gamma/2\text{ml.}$ ; in the case of distearyl lecithin and saponin (Table II), each  $\gamma$  of inhibitor inhibits  $0.17\gamma$  of lysin when the initial quantity is  $200\gamma$ , and  $0.07\gamma$  when the initial quantity is  $80\gamma$ . In the case of serum albumin (Table XI), each  $\gamma$  inhibits from  $0.016\gamma$  to  $0.03\gamma$  according to the initial concentration of saponin, and from  $0.025\gamma$  to  $0.04\gamma$  according to the initial concentration of taurocholate. Each  $\gamma$  of globulin (Ponder, 1944) inhibits about  $0.13\gamma$  of saponin,  $0.15\gamma$  of digitonin,  $0.14\gamma$  of taurocholate, and  $0.22\gamma$  of glycocholate, with a dependence on lysin concentration which has not yet been worked out.

In the case of cholesterol sols, the question as to how many  $\gamma$  of lysin are rendered inert per  $\gamma$  of cholesterol can be answered only if the time of contact between the inhibitor and the lysin, as well as the lysin concentration, is specified. At  $37^\circ\text{C}.$ , and after 24 hours' contact, each  $\gamma$  of cholesterol inhibits from  $5.2\gamma$  to  $8.0\gamma$  of saponin, according to the amount of sol used, when the initial quantity of lysin is  $100\gamma$  (Table VIII); under the same conditions, each  $\gamma$  of cholesterol inhibits about  $12\gamma$  of digitonin when the initial quantity is  $80\gamma$  (Ponder, 1945 b). Cholestryl acetate inhibits about  $1.4\gamma$  of saponin and  $2.0\gamma$  of digitonin, per  $\gamma$  of the ester, under the same conditions of experiment.

The question as to how much of the inhibitory power of plasma can be accounted for by the contained cholesterol (or free cholesterol) is again one which can be answered only if the experimental conditions are defined with respect to initial concentration of lysin, time of contact, and a number of other variables. Reference to Fig. 2 A and B, will show that a quantity of plasma may be either more or less inhibitory than a quantity of cholesterol sol according to whether the time of contact is short or long. Even with the time of contact sufficient for the lysin-inhibitor system to reach equilibrium, the quantity of lysin rendered inert per  $\gamma$  of cholesterol varies with both the initial concen-

tration of lysin and the quantity of cholesterol, and since similar complexities exist in the case of plasma, correlations between the inhibitory power of plasma and the content of total, or free, cholesterol are apt to mean less than they appear to do at first sight.

#### DISCUSSION

So far as the inhibition of hemolysis by plasma is concerned, the principal points of interest have always been (1) whether the inhibition is the result of reactions in the bulk phase of the system or at the red cell surfaces, (2) whether it is possible to develop an expression which will describe the inhibition quantitatively, and (3) an understanding of the extent to which the various components of plasma contribute to the total inhibition. The best way of discussing the results obtained in this paper and in previous papers of this series is to see what light they throw on these questions.

*1. Is the Inhibition Produced by Plasma the Result of Reactions Occurring in the Bulk Phase or at the Cell Surfaces?*—In earlier investigations (Ponder, 1924, 1925) I treated the inhibition produced by serum and plasma as due to a reaction in the bulk phase of the system in which the serum components "react with the lysin in the same sort of way as when reactant A precipitates reactant B from solution." This type of reaction in the bulk phase is what I call a  $\Delta$ -reaction, a quantity  $\Delta$  of the lysin being rendered inert as the result of the combination of the lysin and the inhibitory component. So far as the cholesterol of serum or plasma is concerned, the investigations of Lee and Tsai (1942 a) support this point of view very strongly, for they show that the amount of inhibition depends on the length of time during which the lysin (saponin, digitonin) and a cholesterol sol are allowed to remain in contact, as well as on the temperature.

I have never felt satisfied with this description of the inhibitory effect of plasma, however, as is shown by frequent attempts either to test it further (Ponder, 1932, Ponder and Gordon, 1934) or to abandon it altogether (Ponder, 1943).<sup>16</sup> These attempts had their origin in the difficulties raised by the

<sup>16</sup> The attempt to account for all inhibitory and acceleratory phenomena in terms of  $R$ -values and reactions at the cell surfaces was a step in the wrong direction. If the expressions in terms of  $\Delta$  are complex, those in terms of  $R$  are no less so if the experimental range is sufficiently great (see footnote 10); the observation that the values of  $\Delta$  vary with the number and kind of red cell introduced into the system seems to be accounted for best by a redistribution of lysin within the system, and the supposed similarity between the effects of acceleration and inhibition produced by substances such as the sugars (Ponder and Yeager, 1928, Yeager, 1929) or the monovalent cations (Gordon, 1933) and the inhibitory effect of plasma is only a superficial one. In the case of the former, most of the inhibitory, or acceleratory, effect is produced at the cell surfaces and only a small proportion of it in the bulk phase (Ponder, 1926, Ponder and Yeager, 1928); in the case of the latter, most of the inhibition is produced by  $\Delta$ -reactions in the bulk phase.

experimental observations (*a*) that the values of  $\Delta$  obtained depend not only on the type of red cell introduced into the system but also on the number of red cells, so that the cells do not act as a simple indicator of the amount of lysin  $c_2$  left free after an amount  $\Delta$  has been rendered inert, and (*b*) that the treatment of the results in terms of a  $\Delta$ -reaction exclusively gives rise to awkward expressions (see section 2, below). They were also influenced by the idea that there is an underlying similarity between the inhibitory effects of plasma and those of substances such as the sugars, and a similarity between inhibitory phenomena in general and the phenomenon of acceleration.

The results described in this paper show that although the greater part of the inhibition produced by plasma is the result of a  $\Delta$ -reaction in the bulk phase of the system, the effects of this reaction are modified by redistribution of lysin among the regions of the red cell surface, the bulk phase of the system, and the inhibitory components, and are complicated by some of the plasma components reacting directly with components of the cell surfaces (e.g., the *R*-reactions in the case of the lecithins, and the reactions which influence shape changes in the case of the albumins).<sup>17</sup> In addition, there are enhancing effects among the inhibitory components, probably largely in the bulk phase of the system.

*2. Can a Quantitative Expression for the Inhibition Be Found?*—In 1924 the assumption that the inhibition produced by plasma is due to a  $\Delta$ -reaction in the bulk phase of the system was supported by all the facts then known, although even then it was recognized that the reaction was probably partly reversible. The expression first sought for describing the results of the reaction was one between  $\Delta$  and  $c_2$ , the amount of lysin left free in the system, with the idea that a loose physical compound, rather than a molecular one, was formed. The expression  $\Delta = Ac_2^{1/n}$ , indeed, seemed to be adequate over the limited range explored (Ponder, 1924). The investigations were extended (Ponder, 1925) to cover variations in the amount of inhibitor used, and now the expression had to be modified to  $\Delta = A(c_2 - K)^{1/n}$ , both  $A$  and  $K$  being functions of the quantity of plasma introduced into the systems.

As remarked above, dissatisfaction with this description of the inhibition produced by plasma led to experiments designed to test it further. With the development of improved methods for measuring percentage hemolysis curves it became possible to determine  $\Delta$ , the amount of plasma apparently rendered

<sup>17</sup> The discussion of the mechanism of acceleration and inhibition in which these phenomena are supposed to result from modifications of the cell surface (Ponder, 1941, 1943) applies only to those forms of acceleration and inhibition which are the result of *R*-reactions (most forms of acceleration, inhibition by substances such as the sugars, and possibly by the lecithins and albumins of plasma). In these cases some sort of overall effect, such as a change in the extent to which lysin is concentrated at the cell surfaces, seems to be involved, the surface structure at the same time exhibiting differences in reactivity from point to point, or maybe in a pattern.

inert by the added plasma, at any stage of the hemolytic process, and the results were compatible with the inhibition being the result of a  $\Delta$ -reaction exclusively (Ponder, 1932).<sup>18</sup> Observations were extended to cover times up to 300 minutes (Ponder and Gordon, 1934), and it was established that the value of  $n$  in the expression for the "fundamental reaction" remains unchanged when plasma is added.<sup>19</sup> The expression relating the concentration of lysin and  $\Delta$  was now changed to  $\Delta = \alpha(c_1 - c_{1\infty})^{1/\mu}$ . This gets rid of the arbitrary constant  $K$ , but leaves two other constants,  $\alpha$ , corresponding to the old  $A$ , and  $\mu$ , corresponding to the old  $n$ . The latter seems to be constant for all quantities of plasma used, but the former is a non-linear function of the quantity of plasma. While this expression retains the form of an adsorption isotherm, it is really no better than an empirical formula.<sup>20</sup> The remainder of the paper deals with the idea of competition for lysin between the inhibitor and the red cell surfaces, a subject discussed further in Ponder, 1945 *a*, and in this paper.

The results described in this paper show that the  $\Delta$ -reaction between lysin and plasma in the bulk phase of the system is complicated by competition for lysin between the cell surfaces and the inhibitor, by reactions of plasma components with the cell surfaces, and by enhancing effects of the components upon each other. It is accordingly not surprising that the only expression yet found to describe the inhibition quantitatively should be an empirical one with several arbitrary constants.

### *3. To What Extent Do the Various Components of Plasma Contribute to the*

<sup>18</sup> This test has its practical limitations, because considerable variation in the velocity constant of the fundamental reaction could occur and yet give rise to deviations from the  $\Delta_1/\Delta_2$  relation which would be no greater than the experimental errors would account for. This possibility becomes less as the measurements are made nearer and nearer the asymptotes of the time-dilution curves for 50 per cent and 100 per cent hemolysis respectively, and it is probable that the experimental results obtained constitute a valid test of the point under consideration. The finding that  $\Delta_1/\Delta_2 = 1.0$  would not, however, exclude an  $R$ -reaction in which the initial amount of lysin  $c_1$  was found to behave as if it were of amount  $c_2$ , irrespective of the group of cells in the frequency distribution with respect to which its behavior was tested. Again, the test would not exclude a  $\Delta$ -reaction accompanied by a small variation in the velocity constant, and it should be remarked in this connection that all calculations of the type  $\Delta = c_1 - c_2$ , based on measurements from time-dilution curves, involve the tacit assumption that the velocity constant of the fundamental reaction remains unchanged by the addition of the inhibitor or accelerator. One hopes that this assumption is correct, but in the meantime there is no way of demonstrating its correctness unequivocally.

<sup>19</sup> Some other inhibitors, and some accelerators, change the value of  $n$ . They probably act at the cell surfaces. See Ponder and Yeager, 1930.

<sup>20</sup> It is not always even a good empirical formula. Table XII provides an instance in which  $\Delta$  increases as  $c_1$  decreases.

*Total Inhibitory Effect?*—Although at least four components of plasma have inhibitory properties (cholesterol,<sup>21</sup> globulins, lecithins, and albumins) it is now clear that a question of this kind cannot be answered without further specification. Even in the case of one inhibitory component, the amount of inhibition produced per  $\gamma$  of inhibitor depends on (1) the lysin present in the system, (2) its initial concentration, (3) the pH, (4) the temperature, (5) the order in which the components of the hemolytic system are mixed, (6) the time of contact between lysin and inhibitor in lysin-inhibitor-cell systems, and (7) the kind, and (8) the number, of red cells added to the system. When there is more than one inhibitor present, one has to take account of enhancing effects as well.

Speaking very generally, the inhibitory power of the principal components, expressed as  $\gamma$  of lysin per  $\gamma$  of inhibitor is about 0.1 for the lecithins, 0.2 for the proteins, and 5 to 10 for cholesterol in systems containing saponin, digitonin, or the bile salts. Since the concentration of the plasma proteins is some 50 times that of the free cholesterol in the plasma, the total inhibitory power of the plasma proteins works out as approximately equal to that of the free cholesterol. The enhancing effect of lecithin on the inhibitory effect of cholesterol, and the smaller inhibitory power of the cholesterol esters (if it exists), may combine to increase the fraction of the total inhibitory power which is dependent on cholesterol, but we are still left with a considerable fraction which is dependent on substances other than cholesterol. These calculations, of course, are very rough, for it is not even certain that the sum of the effects of all the inhibitory components adds up to the total inhibitory effect of the plasma; there may be hitherto unidentified inhibitors on the one hand, or the effect of the inhibitors may be partly offset by the effect of accelerators on the other.

It now begins to look as if the use of the same type of method and the interpretation of the results along the same general lines are leading only to increasingly involved situations, and as if our view of the subject were approaching what has been called its horizon of complexity. What is required are methods by means of which the concentration of free lysin in a lysin-cell system can be measured independently after the cells have been added, and by means of which the quantity of lysin combined with an inhibitor such as cholesterol can be determined independently of the course of the hemolytic process. Further analysis would then be possible.<sup>22</sup>

<sup>21</sup> Cholestryl acetate, the only ester hitherto examined for inhibitory power, has about one-fifth the inhibitory effect of cholesterol itself. Until there is further evidence on the point, it cannot be safely assumed that the cholesterol esters of plasma are non-inhibitory.

<sup>22</sup> Collier and Wilbur (1944) have approached the problem of the inhibition of hemolysis by plasma in a somewhat different way. They measure the amount of a

## SUMMARY

This paper contains a description of some of the inhibitory, and occasionally acceleratory, effects of sols of lecithins, cholesterol, and proteins in hemolytic systems containing simple lysins, together with investigations on the nature of the reactions by means of which the effects are brought about. The principal conclusions are:

*A. As regards sols of lecithins.*

1. In lysin-inhibitor-cell systems, distearyl lecithin is an inhibitor of saponin and digitonin hemolysis, part of the effect being the result of a reaction with the components of the red cell surface and part being the result of a reaction with lysin in the bulk phase of the system. Lecithin *ab ovo* (Merck) is an accelerator of saponin hemolysis and either an accelerator or an inhibitor of digitonin hemolysis according to the initial concentration of lysin present in the system. Soybean lecithin is an inhibitor of both saponin and digitonin hemolysis, but both soybean lecithin and lecithin *ab ovo* contain also a hemolytic, or acceleratory, component.

2. The inhibitory effects depend on the order in which the components of the hemolytic system are mixed together. Distearyl lecithin is about 5 times more inhibitory in cell-inhibitor-lysin systems than in lysin-inhibitor-cell systems containing saponin, digitonin, or taurocholate. Lecithin *ab ovo* is more inhibitory in cell-inhibitor-lysin systems when the time of contact between

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lysin (lysolecithin) required to hemolyze a quantity of diluted blood or of washed red cells, assuming that changes in the concentration of lysins, accelerators, or inhibitors in the blood will be reflected in the amount of lysin required. The number of milligrams of added lysin which brings about 50 per cent lysis of 1 ml. of blood or its equivalent in 60 seconds at room temperature is called the antihemolytic value (AHV). The relation between AHV and what I call the inhibitory power of plasma should be that the inhibitory power is proportional to the AHV for whole blood minus the AHV for washed red cells. It should be noticed that the magnitude of the difference between the AHV of system A and that of system B is dependent on the measurement of AHV being made at the arbitrarily selected time 60 seconds, and that if the measurements were made at another time, say 30 seconds, the two AHV values *and their difference* would be different; this is a reflection of the fact that  $\Delta$  is a function of  $c_1$ . Collier and Wilbur's approach to the problem certainly has its advantages. The study of the total inhibitory effect of plasma and its variations is more likely to lead to conclusions of practical interest than is the attempt to reconstruct the total effect from the effects of its parts, particularly as these seem to be non-additive, and the results of the direct measurements, especially when made on systems containing whole blood, have an immediate bearing on the problems of *in vivo* hemolysis. The extent to which it is legitimate to extend the results obtained in dilute *in vitro* systems to *in vivo* systems is very doubtful, especially as not all the components of the *in vivo* systems are known.

cells and inhibitor is short, but when it is long, the hemolytic properties of the lecithin offset its inhibitory properties. A similar state of affairs is observed with soybean lecithin.

3. An increase in temperature decreases the inhibitory effect of distearyl lecithin in systems containing saponin or digitonin.

*B. As regards sols of cholesterol.*

4. The quantity of lysin  $\Delta$  apparently inhibited by a quantity  $Q$  of cholesterol sol is dependent on both the type of red cell and the number of red cells added to the system.

5.  $\Delta$  is a non-linear function of  $Q$  and of  $c_1$ , the initial quantity of lysin present in the hemolytic system,  $\Delta$  generally increasing as  $c_1$  increases.

6. The inhibitory effect of cholesterol sols is essentially due to a reaction between the cholesterol and the lysin in the bulk phase of the system, modified by what appear to be redistribution effects which depend on the kind and number of red cells added to complete the hemolytic system.

7. The value of  $\Delta$  depends on the temperature and on the length of time during which the cholesterol and the lysin remain in contact before the addition of the cells.

8. Distearyl lecithin considerably enhances the inhibitory effects of cholesterol sols.

*C. As regards the proteins.*

9. Freshly prepared serum globulin is inhibitory in systems containing saponin, digitonin, taurocholate, and oleate, and the effect is due to reactions in the bulk phase of the system, modified by redistribution effects.

10. Serum albumin either accelerates or inhibits lysis by saponin, depending on the initial concentration of lysin, and the inhibition depends on such factors as the type of red cell used and the time of contact. In the case of sodium taurocholate, the inhibition has a very marked pH dependence.

*D. As regards plasma.*

11. The way in which the inhibitory effect depends on the length of time during which inhibitor and lysin are in contact before the addition of the cells is not the same when plasma is used as an inhibitor as when a cholesterol sol is used as the inhibitor. The amount of cholesterol sol which is equal in inhibitory power to a given amount of plasma accordingly varies according to the length of the time of contact which is selected.

12. The inhibitory effect in systems containing saponin, plasma, and red cells can be shown to depend on the order in which the components are mixed, when the concentration of the plasma is small.

13. The question as to how much of the inhibitory power of plasma can be accounted for by the contained cholesterol (total or free) is one which can be answered only if the experimental conditions are defined with respect to initial concentration of lysin, time of contact, and several other variables. Very

roughly, about 50 per cent of the total inhibition of plasma, or a little more, can be attributed to the cholesterol fraction.

14. Since the inhibitory effects of plasma are the result of reactions in the bulk phase of the system, complicated by redistributions among the phases, of reactions between some of its components and components of the red cell surface, and of enhancing effects of its components upon each other, it is not surprising that nothing better than an empirical expression should have been found to describe the inhibition quantitatively.

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# THE TOXICITY OF COPPER NITRATE SOLUTIONS TO POLYCELIS NIGRA

## II. THE DUPLEX TOXIC MECHANISM AND THE SYNERGIC EFFECT OF ADDED SOLUTES

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A very large number of investigations have been made of the relationship between the concentration of toxic substances in solution, and the extent of the toxicity produced at different concentrations. The problem has been studied from many viewpoints and the resultant mass of data has been well reviewed by Stiles (1924) and by Clark (1933). The latter, in particular, pays much attention to the variety of mathematical formulae which have been developed to express the toxicity/concentration relationship of toxic solutes—*e.g.* those of Ostwald (1907), Chick (1908), Reichel (1909), Powers (1917, 1920), Porodko (1926), Ponder (1932),—formulae developed as a result of work on plants and animals, living and dead. He comments particularly with regard to these expressions, that there has been virtually no attempt to explain their practical significance.

It seems to the writer that since the same fundamental phenomenon is involved in all this work, *i.e.* the reaction of a toxic substance with a protoplasmic substrate, it would be advisable, where possible, to arrive at some systematic basis whereby the mathematical expressions involved could be used to obtain an understanding of the basic processes at work in any case of toxicity. If this aspect is neglected, the derivation of mathematical formulae can serve no useful purpose.

In the present contribution, it seems best to limit consideration of this aspect to the relationship Survival time/Concentration which has perhaps provided the most reliable previous data. Many cases of toxicity, involving study of this relationship, can be grouped according to the apparent (not necessarily the actual) number of variable factors controlling the dependence of the survival time  $t$  of an organism (or the toxicity as expressed by the reciprocal  $l/t$ ) upon the concentration  $c$  of the toxic substance.

In general, uni-univalent electrolytes, non-toxic organic compounds which kill only in hypertonic solution, and many drugs, give simple survival time/concentration curves expressed by  $t = Kc^p$  where  $K$  and  $p$  are constants and the plot  $\log t/\log c$  gives a straight line. Where such a simple relation holds, the toxicity may be termed "simplex," since the survival time appears to depend

solely and to a constant degree upon the apparently single variable factor, the concentration of toxic solute.

Many substances do not give such a direct relationship between  $t$  and  $c$  over the whole concentration range and in such cases the plot  $\log t/\log c$  used extensively by Clark (1933) and by Gause (1933) appears to be of particular value. The latter author noted for *Paramecium caudatum* in mercuric chloride solutions that the plot  $\log t/\log c$  was a curve consisting of two straight-line sections, each of different slope, merging at a particular concentration. Above this concentration the relation  $t/c$  was expressed by the logarithmic equation

$$\log t = \log K + \varphi \log c$$

with particular values for  $K$  and  $\varphi$ ; and below it by a similar type of equation but with quite different values for the two constants.

Szucs (1912) noted a case where the toxicity of concentrated solutions was greater than that expected from the results with dilute solutions, and if the data given by Chick (1908) for the action of phenol on *B. paratyphosus*, by Carpenter (1927) for the killing of *Leuciscus phoxinus* by lead nitrate or mercuric chloride, or by Ing and Wright (1932) for the paralysis of frog's sartorius muscle by quaternary ammonium iodides, are replotted logarithmically, the plot  $\log t/\log c$  gives, in each case, a curve composed of two linear portions. In each case, the nature of the ratio  $t/c$  is similar for all concentrations, but the degree of the relationship differs in the two sections of the concentration range.

Where this occurs, if it be assumed that over one of the two sections of the concentration range  $t$  is dependent on only the one variable factor  $c$ , then the different degree of dependence of  $t$  upon  $c$  evident for the other section can be explained as due to the introduction of a second variable factor. Since the toxicity in such cases appears to depend on the existence of two independent variable factors, it is convenient to term the mechanism "duplex" to distinguish it from the "simplex" toxicity outlined above.

Many examples of toxicity do not fall into either of these categories, the  $\log/\log$  plot being non-linear. Although this is often due to the fact that the concentration ranges examined have been too limited to permit analysis of the results, such cases must be left outside the scope of this contribution. The subdivision used above is, of course, tentative, but it does enable such analysis of a large section of toxicity data as might, in many cases, aid in determining the number of variable factors controlling the toxicity. Such an estimate would appear to be essential to any investigation of the nature of those factors.

The writer<sup>1</sup> (1939) showed that the killing of *Polyclis nigra* by copper nitrate solutions was a case of what is here termed "duplex" toxicity, the relationship between  $t$  and  $c$  over a wide range of concentration being given by the expression  $t = Kcp$ . For hypertonic solutions of copper nitrate  $K$  and  $\varphi$  had each a certain

<sup>1</sup> Paper I of this series.

constant value, and for hypotonic solutions the constants had quite different values, the degree of toxicity of hypertonic solutions being relatively much greater than that of hypotonic solutions. In consequence, the plot  $\log t/\log c$  was a duplex curve with two linear sections merging at a concentration approximating the isotonic point of normal tissue fluids. In concentrations above this point the organisms were observed to shrink and invaginate; in lower concentrations the epidermal cells cytolyzed presumably as a result of water intake.

The coincidence of this behaviour, resulting from osmotic water exchange, with the abrupt change in the degree of toxicity of the solutions, led to the suggestion that whereas the basic toxicity depended on the concentration of copper nitrate present, the relatively high toxicity of the hypertonic solutions might be due to the introduction of a second variable lethal factor, the osmotic withdrawal of water, which would be operative only in hypertonic solutions. It was indicated that this could be tested by investigating the toxicity of a range of copper nitrate solutions, the osmotic pressures of which were adjusted to a single constant value by the addition of inert solutes, thus eliminating the osmotic variable. The present contribution describes an investigation along these lines of the factors responsible for the duplex toxicity of copper nitrate solutions toward *Polycelis nigra*.

In the course of interpreting the results of this new investigation with added solutes, it was observed that the mixtures of copper nitrate plus added solutes displayed toxic effects greater than would be expected from consideration of their separate toxicities.

The phenomenon of antagonism, in which a mixture of two toxic solutes displays a much lower toxicity than would be anticipated from consideration of the separate toxicities of the components of the mixture, has received much attention and can be regarded as well authenticated. The opposite phenomenon of synergy, in which the combined toxicity of the two toxic solutes is markedly greater than would be expected, is also well known in the case of organic drugs and is of some importance in pharmaceutical practice. It seems, however, that it has been rarely observed for mixtures of metallic salts and, except for the cases described by Lipman (1909), Loeb (1916), Raber (1917), and Osterhout (1922), there is little evidence in the literature that the toxicity of a mixture of metallic salts can be greater than the sum of their combined toxicities. In fact, the extent of the literature on antagonism would suggest that the opposite is generally the case.

It is of interest, therefore, to record such a case of synergy as occurring in the copper nitrate + sodium chloride and also in the copper nitrate + glucose mixtures used in the present investigation.

#### EXPERIMENTAL

The technique used in this work for the standardisation of the organisms and observation of their behaviour and survival times in the toxic solutions was

described in detail in Paper I (White, 1939). All experiments were carried out in duplicate with 100 ml. of solution at a temperature of  $23^{\circ}\text{C.} \pm 1^{\circ}\text{C.}$  Data regarding the osmotic pressures of the glucose solutions used were obtained from

TABLE I

*Survival Times of Polycelis nigra in Copper Nitrate Solutions of the Concentrations and Osmotic Pressures Stated*

Temperature =  $25^{\circ}\text{C.}$

Concentration $c$	Osmotic pressure	Survival time $t$
$N$	atm.	min.
0.0001	0.0037	432.25
0.0005	0.018	251.25
0.001	0.036	180.0
0.005	0.178	92.25
0.01	0.349	67.50
0.05	1.63	32.25
0.10	3.11	19.0
0.14	4.32	14.75
0.20	6.01	6.25
0.30	8.71	0.80
0.40	11.47	0.33
0.50	14.01	0.23

TABLE II

*Survival Times of Polycelis nigra in Glucose Solutions and in Sodium Chloride Solutions of the Concentrations and Osmotic Pressures Stated*

Temperature =  $23^{\circ}\text{C.}$

Concentration $c$	Glucose		Sodium chloride		
	Osmotic pressure	Survival time $t$	$N$	Osmotic pressure	Survival time $t$
$x$	atm.	min.			
2.0	62.2	3.1	2.0	94.2	0.34
1.5	43.2	4.83	1.5	70.0	0.50
1.0	27.1	10.25	1.0	46.1	1.0
0.75	20.3	21.0	0.75	34.3	2.5
0.50	12.8	49.5	0.50	22.7	5.0
0.40	10.2	81.75	0.40	18.3	16.25
0.30	7.7	152.25	0.30	13.9	28.0
0.20	5.0	—	0.20	9.2	—

International critical tables (1928 a). Similar data were calculated (using the equation  $PV = iRT$ ) for sodium chloride solutions from the freezing point data given in International critical tables (1928 b) for such solutions, and for copper nitrate solutions from conductivity data available in the same source (1928 c).

The survival times of *Polycelis nigra* in copper nitrate solutions of concentrations 0.0001 N to 0.5 N were then reinvestigated, the results confirming the findings reported in Paper I. Table I details the concentrations investigated, their osmotic pressures, and the survival times of the organisms at each concentration.

For comparative purposes the survival times of *Polycelis* in a range of sodium chloride and of glucose solutions of known osmotic pressures were also determined and the results, with the concentrations and osmotic pressures are listed in Table II.

TABLE III

*Survival Times of Polycelis nigra in Copper Nitrate Solutions Adjusted to an Osmotic Pressure of Approximately 14 Atmospheres by the Addition of Glucose*

Temperature = 23°C.

Copper nitrate N	+ Glucose		Survival time <i>t</i> in mixtures <i>min.</i>
	Concentration <i>c</i> M	Osmotic pressure atm.	
0.5	—	—	0.21
0.4	0.104	2.52	0.35
0.3	0.21	5.3	0.62
0.2	0.32	8.2	1.4
0.14	0.39	10.0	2.27
0.10	0.44	11.25	5.35
0.05	0.50	12.8	11.25
0.01	0.553	14.5	13.75
0.005	0.558	14.55	17.25
0.001	0.563	14.57	24.0
0.0005	0.564	14.6	30.25
0.0001	0.564	14.6	45.0

Two series of copper nitrate solutions of the same concentrations as those listed in Table I were then set up and the solutions of each series adjusted to a constant osmotic pressure of approximately 14 atmospheres (that of 0.5 N copper nitrate solution, the highest concentration used) by adding to one series the calculated amounts of glucose (Table III), and to the other series the calculated amounts of sodium chloride (Table IV), required to bring about the adjustment. The survival times of *Polycelis* in each series are given in the respective tables. It must be noted here that the mixed solutions were so made up as to contain a final concentration of each solute corresponding to that given in the tables, not by mixing solutions of those concentrations.

Finally, the effect of osmotic water intake was investigated by adjusting a series of hypotonic copper nitrate solutions of concentrations 0.0001 N to 0.10 N to a constant osmotic pressure of approximately 3.2 atmospheres (slightly

below isotonicity) by the addition of glucose. Table V gives details of these solutions and the corresponding survival times.

TABLE IV

*Survival Times of Polycelis nigra in Copper Nitrate Solutions Adjusted to an Osmotic Pressure of Approximately 14 Atmospheres by the Addition of Sodium Chloride*

Temperature = 23°C.

Copper nitrate <i>N</i>	+ Sodium chloride		Survival time <i>t</i> in mixtures
	Concentration <i>c</i>	Osmotic pressure	
0.5	—	—	0.23
0.4	0.055	2.3	0.33
0.3	0.113	5.2	0.54
0.2	0.175	7.9	0.68
0.14	0.215	9.9	1.0
0.10	0.245	11.25	2.25
0.05	0.280	12.8	5.0
0.01	0.30	13.8	7.5
0.005	0.305	14.0	11.0
0.001	0.310	14.1	14.0
0.0005	0.312	14.3	20.0
0.0001	0.312	14.3	20.0

TABLE V

*Survival Times of Polycelis nigra in Copper Nitrate Solutions of Concentrations up to 0.1 N Adjusted to Approximate Isotonicity by the Addition of Glucose*

Temperature = 23°C.

Copper nitrate	+ Glucose		Survival time <i>t</i> in mixtures
	Concentration <i>c</i>	Osmotic pressure	
<i>N</i>	<i>M</i>	atm.	min.
0.1	—	—	19.1
0.05	0.06	1.5	29.0
0.01	0.113	3.0	68.0
0.005	0.119	3.1	95.1
0.001	0.126	3.17	178.5
0.0005	0.128	3.2	253.0
0.0001	0.128	3.2	430.3

In conformity with the findings in Paper I, in the experiments giving the data in Table I (*i.e.* where the normal osmotic pressure of the copper nitrate solution remained unaltered) cytolysis of the organisms occurred in all the hypotonic solutions. Similarly, in all the slightly hypotonic solutions listed in Table V,

a sparse cytolysis was observed. In contrast to this, no cytolysis occurred in the glucose or sodium chloride solutions, nor in those copper nitrate solutions the osmotic pressure of which had been brought up to 14 atmospheres by the addition of glucose or of sodium chloride. The invagination previously observed only in the hypertonic solutions of copper nitrate now characterised all the solutions listed in Tables III and IV, and can therefore be due only to osmotic withdrawal of water. The conclusion that cytolysis results from water intake in hypotonic solutions thus appears to be justified.

The fact that the organism undergoes osmotic water exchange in the toxic solutions having thus become evident, it remains to consider the significance of the data accumulated in these experiments, a task which is most easily carried out by discussing the curves obtained.

#### DISCUSSION

From Table II, giving details of the survival times of *Polycelis* in solutions of glucose of concentrations 0.2 M to 2.0 M, and of sodium chloride of concentrations 0.2 N to 2.0 N, it is clear that similar concentrations of glucose and of sodium chloride do not show comparable toxicities, the values for sodium chloride being much higher than those for glucose. The resemblance of the survival times for the two solutes is closer however (although by no means complete) if solutions of comparable osmotic pressures are compared. Since, for each solute, concentrations below 0.2 M were non-lethal, it seems logical to conclude that the toxicity of these solutions is due mainly to their osmotic withdrawal of water from the organisms. Jones (1937) also concluded that the lethal effect of sodium chloride to *Polycelis* "is primarily due to withdrawal of water from the body."

In Fig. 1, where the logarithms of the survival times are plotted against the logarithms of the concentrations of each of the toxic solutions and mixtures used, it can be seen that the logarithmic curves for both glucose and sodium chloride are linear. In each case, over the whole concentration range, the survival time bears to the concentration a constant relationship given by the expression  $t = Kc^p$ . Both glucose and sodium chloride exhibit a simplex toxicity, the same fundamental toxic process being operative over the whole lethal concentration range, a fact which is in keeping with the earlier assumption that the lethal effect of both solutes is almost purely osmotic. This generalisation is but little affected by the fact, evident from Table II, that sodium chloride solutions although killing mainly in virtue of their osmotic effect are somewhat more toxic than glucose solutions of comparable osmotic pressures.

Comparison of Tables I and V shows further, that the adjustment of hypotonic copper nitrate solutions to approximate isotonicity by the addition of glucose caused no change in the toxicity of these solutions as compared with their normal toxicity. It is clear from this that the osmotic water intake which

characterises these hypotonic solutions does not, therefore, change their lethal effect which must be due solely to the presence of the copper salt.

The plot  $\log t/\log c$  in copper nitrate solutions given in Fig. 1 agrees with that in Paper I in being duplex, with two linear sections merging at the concentration 0.11 N. Evidently, if the factor responsible for the relatively high toxicity of the hypertonic solutions were to be eliminated, or alternatively controlled by maintaining its effect at a constant level in each concentration, this logarithmic survival curve would become simplex in character; *i.e.*, it would be a straight

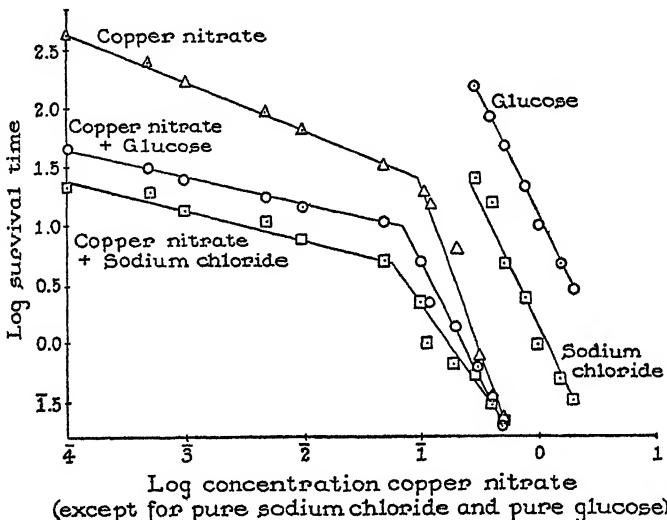


FIG. 1. Logarithmic survival curves for *Polycelis nigra* in solutions of glucose; sodium chloride; copper nitrate; and of copper nitrate + glucose and copper nitrate + sodium chloride mixtures made up to have an osmotic pressure of 14 atmospheres. Temperature = 23°C.

line with a constant relationship between the survival time and the concentration for each concentration, as in the case of glucose and sodium chloride.

The remaining curves in Fig. 1 depict logarithmically the relationship  $t/c$  in copper nitrate solutions of the same concentration range as that already discussed but with the osmotic pressures controlled at a constant value as described previously—in the one case by glucose, in the other by sodium chloride. It is immediately clear that control of the osmotic factor has not eliminated the duplex nature of the toxicity, for each of these curves is again composed of two sections. From this result it is quite evident that whereas hypertonic copper nitrate solutions are more toxic than would be expected from consideration of the toxicity of hypotonic solutions, this greater degree of toxicity cannot be due to the osmotic withdrawal of water which characterises these solutions.

This conclusion is borne out by the fact that the order of toxicity due to osmosis (as instanced in Table II) is far too small to account for the great increase of toxicity displayed by the hypertonic copper nitrate solutions. This latter is important in that it rules out any possibility that the present findings might be invalidated by the fact that mixtures of solutes do not always display a total osmotic effect equivalent to the sum of their separate effects.

Since it has already been shown that the osmotic water intake found in the hypotonic solutions does not affect the survival times it must be concluded that osmosis is not an important factor in the duplex toxicity of copper nitrate solutions, despite the fact that the change in the degree of toxicity corresponds closely with the change from hypotonic to hypertonic solutions.

It was shown earlier that duplex toxicity is by no means confined to the present case and it still remains to explain why, in many cases, the toxicity of solutions of high concentrations is relatively much greater than would be expected from data applying to solutions of low concentrations. The present work does no more than prove that the change of toxicity in the case of copper nitrate is not due to any osmotic effect and, in fact, analysis of the data of other workers shows that the change of toxicity need not coincide with the isotonic point of the toxic solutions.

It is possible that the explanation of the phenomenon lies in the field of protein chemistry rather than in that of the physical chemistry of solutions, and it would certainly be helpful if more data were available concerning the extent of absorption of cations and anions by living organisms from high and low concentrations of toxic salts. The literature, however, appears to contain nothing that can be directly applied to the present problem, and the scarcity of quantitative data along these lines makes speculation useless. It can only be emphasised that the factors responsible for duplex toxicity must be identified before any approach to an understanding of their working can be even moderately successful.

Quite apart from this problem, it remains to discuss the synergy mentioned in the introductory section as having been observed in the course of the investigation discussed above. In the course of interpreting the results of the work already detailed, it was felt desirable to compare the observed toxicities of the mixtures used with their expected toxicities, as calculated from the observed toxicity data for each of the solutes.

Addition of the observed toxicity (*i.e.* the reciprocal  $l/t$ ) of any particular concentration of copper nitrate to the toxicity of the sodium chloride (or glucose) concentration used to bring the osmotic pressure of the mixture to the standard value of 14 atmospheres, gave the expected toxicity of that particular mixture. This was done for all the copper nitrate + sodium chloride and copper nitrate + glucose mixtures used in the above work. In support of the general conclusions already outlined, it may be stated that curves plotted from

these calculated data are duplex in type, conforming with the general features of the curves based on the observed results.

Here, however, the similarity ends. Comparison of the expected toxicities of the mixtures with the observed values showed that, in general, the mixtures were far more toxic than would be expected from consideration of the separate toxicities of the components. This is shown by Table VI where it may be seen that the observed toxicity of a mixture may be as much as twelve times its expected value.

In both series of mixtures, at each extreme of the copper nitrate concentration range, where the toxic effect of one solute is small relative to that of the other,

TABLE VI  
*Illustrating the Synergy Observed in Copper Nitrate + Glucose and in Copper Nitrate + Sodium Chloride Mixtures*

Concentration <i>c</i>		Toxicity 1000/t		Ratio Tox. obs. Tox. exp.	Concentration <i>c</i>		Toxicity 1000/t		Ratio Tox. obs. Tox. exp.
Cu(NO <sub>3</sub> ) <sub>2</sub>	Glucose	Ob-served	Ex-pected		Cu(NO <sub>3</sub> ) <sub>2</sub>	NaCl	Ob-served	Ex-pected	
<i>n</i>	<i>m</i>				<i>n</i>	<i>n</i>			
0.5	—	4672	4348	1.09	0.5	—	4444	4348	1.02
0.4	0.104	2837	3334	0.86	0.4	0.055	3077	3334	0.92
0.3	0.21	1613	1254	1.29	0.3	0.113	1852	1254	1.48
0.2	0.32	714	168	4.24	0.2	0.175	1491	170.2	8.70
0.14	0.39	485	80	6.50	0.14	0.215	1000	84.4	11.85
0.10	0.44	187	68	2.74	0.10	0.245	444	75.5	5.88
0.05	0.50	89	51	1.74	0.05	0.280	200	63.4	3.15
0.01	0.553	72.7	38.2	1.90	0.01	0.30	133.3	51.1	2.61
0.005	0.558	48	34.8	1.66	0.005	0.305	90.9	49.7	1.85
0.001	0.563	41.6	29.7	1.40	0.001	0.310	71.4	45.8	1.56
0.0005	0.564	33	29	1.14	0.0005	0.312	50	45.2	1.11
0.0001	0.564	22.2	27.3	0.81	0.0001	0.312	50	43.5	1.15

the divergence of the observed from the expected toxicity is negligible. Where, however, the concentrations are such that both components of the mixtures are exerting a more equal toxic effect, the ratio of the observed to the expected toxicity is much greater than unity. The degree of divergence is so marked as to be well beyond the limit of experimental error, and the only possible explanation is that the mixtures display a marked degree of synergy.

It would be of little advantage at this stage to attempt to discuss the basis of this synergic effect—the phenomenon is not one which the original experiments were designed to bring to light and clearly, further work is called for along this line. Modern theories of the behaviour of electrolytes in solution provide for anomalous behaviour in the case of strong solutions or mixtures of electrolytes and electrolyte + non-electrolyte mixtures, but the order of such anomalies is in no way comparable with the twelvefold increase of toxicity ob-

served for one of the mixtures used in this work. The present contribution must content itself with recording this case of synergy and must leave the question of explanation until it can be accompanied by further data.

#### SUMMARY

The estimation of the number of variables operating in cases of toxic action toward living organisms is discussed. The toxicity of glucose, sodium chloride, and copper nitrate solutions to *Polycelis nigra* has been investigated and also that of copper nitrate solutions whose osmotic pressure was adjusted to a constant level by the addition of glucose or of sodium chloride.

It is shown that hypertonic solutions of copper nitrate are abnormally toxic but that the osmotic variable is not the factor responsible for this abnormally high toxicity. The lack of data which might elucidate such problems is indicated.

During the course of this work it was observed that mixed solutions of copper nitrate + sodium chloride and copper nitrate + glucose exhibit toxicities greater than those expected from consideration of the separate toxicities of the components of the mixtures.

The author is deeply indebted to Professor R. D. Laurie, M.A., and Professor T. C. James, D.Sc., of the Zoology and Chemistry Departments of the University College of Wales, and to Professor W. Stiles, F.R.S., of the University of Birmingham Botany Department for their interest and help in this problem.

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## EFFECTS OF HYDROXYL ON NEGATIVE AND POSITIVE CELLS OF *NITELLA*

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As  $\text{OH}^-$  has unique properties its action on protoplasm is of especial interest.

There appears to be little change in the potential of *Nitella* when the external pH is raised from 5 to 9 but interesting results are obtained by applying 0.01 M NaOH or KOH. The effects are reversible if the exposure is not too prolonged.<sup>1</sup>

With KOH we find<sup>2</sup> that with some cells the more dilute solution is positive to the more concentrated solution (positive cells) but with others the more dilute solution is negative (negative cells): the latter can be converted to the former by sufficient exposure to KOH.

This might be accounted for on the ground that the protoplasm contains an organic acid which makes the cell negative and which is dissolved out by KOH.

<sup>1</sup> The application of 0.01 M NaOH or KOH was not toxic if the exposure did not exceed 20 minutes and the same cell could be used in further experiments on subsequent days. It should be remembered that only a small portion of the cell (about 2 cm.) was covered by the reagent. When the whole cell was covered the effect was greater but when cells were entirely submerged in 0.01 M NaOH half the cells lived 6 hours and in 0.01 M KOH 4 hours.

<sup>2</sup> The cells, after being freed from neighboring cells, stood in the laboratory at 15°C.  $\pm$  1°C. in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, 17, 87) for several days.

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, 21, 541). Temperature 20-26°C. Regarding the amplifier see the reference just cited.

Two spots on the cell, *A* and *B*, were connected to a spot *C* through a recording galvanometer. At the end of the experiment, *A*, *B*, and *C* were killed (in this order) by applying chloroform, which reduced the P.D. at each spot to zero. During the experiments the solutions at *A* and *C* were changed but at *B* the solution was not disturbed: this was a relatively dilute solution.

It was then possible to ascertain the total potential across the protoplasm at *A* and *B* at any previous point on the record on the assumption that *C* had remained constant up to that point (or by correcting for any change). If *C* had changed the amount of alteration could be measured because it would appear as a simultaneous change at *A* and *B* (in the same direction at both).

We find that under certain conditions the mobility of  $\text{OH}^-$  may be intermediate between that of  $\text{K}^+$  and  $\text{Na}^+$ : hence the protoplasmic surface cannot be a pore system for in such a structure all cations must have a higher mobility than all anions or *vice versa*.

We may now consider some important measurements and their significance.

*Concentration Effect of NaOH.*—Measurements of the concentration effect were made in order to calculate the relative mobilities of the ions in the outer protoplasmic surface. This surface may be called  $X$  for convenience.

When 0.001 M NaOH in contact with *Nitella* is replaced by 0.01 M NaOH the P.D. becomes more positive.<sup>3</sup> This indicates that the mobility of  $\text{OH}^-$  in  $X$  is greater than that of  $\text{Na}^+$ . In this respect  $\text{OH}^-$  differs from the anions mentioned in previous studies on *Nitella* since they have in all cases had lower

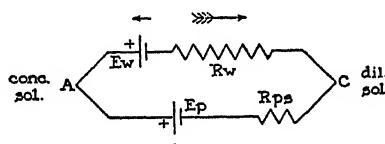


FIG. 1. Diagram of circuit in a negative cell when  $A$  is in contact with a concentrated solution of KOH and  $C$  is in contact with a more dilute solution of KOH. The diffusion potential in the cellulose wall is  $E_w$  and that in the protoplasm is  $E_p$ . The resistance in the cellulose wall is  $R_w$  and that in the protoplasm and sap is  $R_{ps}$ .

The direction of the E.M.F. of  $E_w$  and of  $E_p$  is shown by the small arrows. The direction of the current around the circuit is shown by the large feathered arrow. The dilute solution is negative in the external circuit: this is true whether  $E_p$  is greater than  $E_w$  or *vice versa* and hence it does not depend on the direction of the large feathered arrow.

mobilities<sup>4</sup> than  $\text{Na}^+$ . As already stated, there is ground for thinking that  $X$  contains an acid: this we may call  $\text{HA}$  and assume that the entering NaOH may be changed to  $\text{NaA}$  by the reaction  $\text{NaOH} + \text{HA} = \text{NaA} + \text{H}_2\text{O}$ . Hence we may be dealing chiefly with  $v_A$  rather than with  $v_{\text{OH}}$  in  $X$ . It is, however, more convenient to speak of  $v_{\text{OH}}$  with the understanding that it includes  $v_A$  and this will be done in the subsequent discussion.

When the cell is in contact with 0.001 M NaOH at two spots,  $A$  and  $C$ , the diffusion potentials in  $X$  at  $A$  and  $C$  are equal and opposite and the P.D. between these spots is zero or nearly so. After allowing several minutes for diffusion to occur in  $X$  and in the cellulose wall we replace 0.001 M NaOH at  $A$  by 0.01 M NaOH and thus increase the diffusion potentials at  $A$ . The increase in  $X$

<sup>3</sup> The potential is called positive when the positive current tends to flow from the sap across the protoplasm to the external solution.

<sup>4</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 715.

may be called  $E_p$  and that in the cellulose wall  $E_w$ . We may assume that the new concentration gradient in the cellulose wall is between 0.01 M and 0.001 M NaOH and that in  $X$  is between  $S$  (0.01 M) NaOH and  $S$  (0.001 M) NaOH, where  $S$  is the partition coefficient of NaOH.<sup>5</sup>

A simplified diagram of the electrical circuit is shown in Fig. 1. The only potentials which appear in the diagram are  $E_w$  and  $E_p$ , both of which may be regarded as located at  $A$ . The potentials at other places may be supposed to cancel out.

The current  $I$  is given by

$$I = \frac{E_p - E_w}{R_w + R_{ps}} \quad (1)$$

Here  $R_w$  is the resistance in the cellulose wall and  $R_{ps}$  that in the protoplasm and sap.

For the P.D. between  $A$  and  $C$  we have

$$\text{P.D.} = E_p - IR_{ps} \quad (2)$$

By substitution and rearrangement we obtain from (1) and (2)

$$\text{P.D.} = \frac{E_p R_w + E_w R_{ps}}{R_w + R_{ps}} \quad (3)$$

On the basis of rough calculations we assume that  $R_w \div R_{ps} = 11$ . This is a larger value than is indicated by the experiments of Blinks<sup>6</sup> but in the present case there were two regions between  $A$  and  $C$  where the cell was surrounded by air (to prevent short-circuiting). This gave an opportunity for the cell wall to dry out which would cause the salts in the cellulose wall to move<sup>7</sup> into  $X$  thus increasing the resistance of the cellulose wall and decreasing that of  $X$ . The cellulose wall between  $A$  and  $C$  was imbibed with tap water or with Solution A.

If we assume a lower value for  $R_w \div R_{ps}$  the calculated value of the concentration effects of NaOH and of KOH may become unduly high.

Since absolute values are not needed we may write  $R_w = 11$  and  $R_{ps} = 1$ .

Taking the ionic mobilities in water as proportional to ionic conductivities we may write<sup>8</sup> for 25°C.  $\mu_{\text{Na}} = 50.1$  and  $\nu_{\text{OH}} = 198$ . Assuming that these

<sup>5</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1943-44, 27, 91. In calculations we employ the activity partition coefficient which is constant.

<sup>6</sup> Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 495.

<sup>7</sup> This is not only by simple diffusion but also by the forces which cause salts to move in and reach a higher concentration inside than outside.

<sup>8</sup> See MacInnes, D. A., *The principles of electrochemistry*, New York, Reinhold Publishing Corporation, 1939, p. 342.

values hold for the cellulose wall which is imbibed with aqueous solution we have (at 25°C.)

$$\begin{aligned} E_w &= 59 \frac{v_{OH} - u_{Na}}{v_{OH} + u_{Na}} \log \frac{a_1}{a_2} \\ &= 59 \frac{198 - 50.1}{198 + 50.1} \log \frac{0.01(0.90)}{0.001(0.99)} \\ &= 34 \text{ mv.} \end{aligned}$$

here  $a_1$  and  $a_2$  are the activities in water<sup>9</sup> (at 25°C.).

Replacing 0.001 M NaOH at  $A$  by 0.01 M NaOH gave a change in a positive direction of 36 mv.<sup>10</sup>

Substituting values in Equation 3 we have

$$36 = \frac{E_p(11) + 34(1)}{11 + 1}$$

whence  $E_p = 36.2$  mv.

We may calculate the relative mobilities in  $X$  by the usual formula (for 25°C.).

$$E_p = 36.2 = 59 \frac{v_{OH} - u_{Na}}{v_{OH} + u_{Na}} \log \frac{a_1}{a_2}$$

where  $a_1 \div a_2$  is the ratio of ionic activities of NaOH in  $X$  which is taken as equal to the ratio of concentrations in the external solution (as in previous papers<sup>11</sup>). Putting  $u_{Na} = 1$  we obtain  $v_{OH} \div u_{Na} = 4.2$  (in water  $v_{OH} \div u_{Na} = 198 \div 50.1 = 3.95$ ).

These calculations, like most others in this paper, may be regarded as merely approximate.

It will be shown later that the concentration effect of NaOH can be reversed by exposure to KOH (p. 52).

*Concentration Effects of KOH.*—When we apply KOH to cells not previously subjected to experimental treatment we find that with some the dilute solution is negative to the more concentrated (as with NaOH). These may be called negative cells for convenience. We also find some which behave in the opposite fashion, with dilute solution positive; these may be called positive cells. In

<sup>9</sup> MacInnes, D. A., *The principles of electrochemistry*, New York, Reinhold Publishing Corporation, 1939, p. 155.

<sup>10</sup> This is usually reversible; *i.e.*, when the opposite change is made the P.D. changes in the opposite direction by approximately the same amount. But when the change is in a negative direction an action current may result, giving too large a value.

<sup>11</sup> It has been found in studying models that the ratio of concentrations in the external solutions is approximately the ratio of activities in the non-aqueous phase. Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1942-43, 26, 293.

the same lot both positive and negative cells may occur. The reason for this will be discussed later (p. 52).

The values for negative cells are as follows. Taking, as before, conductivities in water<sup>8</sup> as proportional to mobilities we may for convenience (at 25°C.) put  $u_K = 73.5$  and  $v_{OH} = 198$ .

Hence when 0.001 m KOH at *A* is replaced by 0.01 m KOH the change in the cellulose wall is<sup>12</sup>

$$E_w = 59 \frac{198 - 73.5}{198 + 73.5} \log \frac{0.01(0.90)}{0.001(0.99)}$$

$$= 26 \text{ mv.}$$

On replacing 0.001 m KOH at *A* by 0.01 m KOH the average change was 44 mv. in a positive direction.

Inserting these values in Equation 3 we have

$$44 = \frac{E_p(11) + 26(1)}{11 + 1}$$

whence  $E_p = 45.6$  mv.

We may now write for the mobilities in *X* (see p. 46)

$$45.6 = 59 \frac{v_{OH} - u_K}{v_{OH} + u_K} \log \frac{a_1}{a_2}$$

whence (taking  $a_1 \div a_2 = 10$ ) we have  $v_{OH} \div u_K = 7.8$  (in water we have  $v_{OH} \div u_K = 198 \div 73.5 = 2.7$ ).

If all the mobilities were constant we could calculate  $u_K \div u_{Na}$  but we find that they are not constant in cells exposed to OH.

In positive cells, 0.001 m KOH is positive to 0.01 m KOH and the current has the direction shown in Fig. 2. We have

$$I = \frac{E_p + E_w}{R_w + R_{ps}} \quad (4)$$

For the P.D. between *A* and *C* we have

$$\text{P.D.} = E_p - IR_{ps} \quad (5)$$

By substitution and rearrangement we obtain from (4) and (5)

$$\text{P.D.} = \frac{E_p(R_w) - E_w(R_{ps})}{R_w + R_{ps}} \quad (6)$$

In a typical lot of cells the change in a negative direction when 0.001 m KOH was replaced by 0.01 m KOH was 46 mv.

Hence we have

$$46 = \frac{E_p(11) - 26}{11 + 1}$$

<sup>12</sup> The activity coefficients are here taken as equal to those for NaOH, see p. 46.

whence  $E_p = 52.5$ .

For the mobilities in  $X$  we have

$$52.5 = 59 \frac{u_K - v_{OH}}{u_K + v_{OH}} \log \frac{a_1}{a_2}$$

Proceeding as with NaOH for mobilities in  $X$ , we obtain  $u_K \div v_{OH} = 17.2$ .

*Concentration Effects of NaCl and of KCl.*—Here the dilute solution is always positive indicating that  $v_{Cl}$  is less than  $u_{Na}$  or  $u_K$ . Taking, as before, the ionic

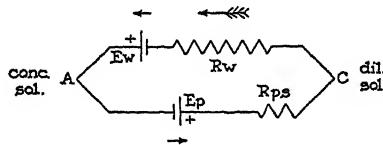


FIG. 2. Diagram of circuit in a positive cell when  $A$  is in contact with a concentrated solution of KOH and  $C$  is in contact with a more dilute solution of KOH. The diffusion potential in the cellulose wall is  $E_w$  and that in the protoplasm is  $E_p$ . The resistance in the cellulose wall is  $R_w$  and that in the protoplasm and sap is  $R_{ps}$ .

The direction of the E. M. F. of  $E_w$  and of  $E_p$  is shown by the small arrows. The direction of the current around the circuit is shown by the large feathered arrow. The dilute solution is positive in the external circuit. This is true only when  $E_p R_w > E_w R_{ps}$ , for when the P.D. is zero we have

$$\text{P. D.} = 0 = \frac{E_p R_w - E_w R_{ps}}{R_w + R_{ps}}$$

$$0 = E_p R_w - E_w R_{ps}$$

$$\frac{E_p}{E_w} R_w = R_{ps}$$

mobilities as proportional to the ionic conductivities we may write (at 25°C.) for  $E_w$  in the case of NaCl.

$$E_w = 59 \frac{76.3 - 50.1}{76.3 + 50.1} \log \frac{0.01(0.90)}{0.001(0.99)}$$

$$= 11.7 \text{ mv.}$$

Replacing 0.01 M NaCl by 0.01 M NaCl gives a change in a negative direction of 29 mv.

Hence we have

$$29 = \frac{E_p(11) - 11.7}{11 + 1}$$

whence  $E_p = 32.7$ .

For the mobilities in  $X$  we obtain  $u_{Na} \div v_{Cl} = 3.5$ .

by 0.01 M NaOH the P.D. becomes more positive. The average value<sup>14</sup> in one lot of cells was 120.<sup>10</sup> In another lot it was 89.

If we calculate partition coefficients by means of Henderson's equation, as in former papers,<sup>15</sup> we obtain a high value for  $S_{\text{NaOH}}$  (the concentration of NaOH in  $X$  divided by that in the external solution) but as this involves mobilities which may not be constant in cells exposed to OH the quantitative significance of  $S_{\text{NaOH}}$  is doubtful. A high value for  $S_{\text{NaOH}}$  is not surprising if  $X$  contains an organic acid as will appear later (p. 54).

Let us now consider similar experiments with KOH. With negative cells (where the dilute solution is negative with KOH) we find that replacement of 0.01 M KCl by 0.01 M KOH gives a change in a positive direction of 145 mv.<sup>10</sup>

Although it is not feasible to calculate the partition coefficient of KOH (because mobilities are not constant) it is evident that it is greater than that of KCl. For when 0.01 M KCl is replaced by 0.01 M KOH the change in P.D. occurs promptly but when the opposite change is made the alteration of P.D. is very slow. Similar considerations apply to NaCl and NaOH.

When we turn to positive cells (which show dilute solution positive with KOH) we see a different picture. On replacing 0.01 M KCl by 0.01 M KOH the change in P.D. is small and may be in a positive or in a negative direction. It would thus appear that the effects of KCl resemble those of KOH in these cells.

When 0.01 M NaCl is replaced by 0.01 M KOH we might expect a change in a negative direction (since  $\mu_K$  is greater than  $\mu_Na$ ) unless the value of  $v_{\text{OH}}$  is so much greater than that of  $\mu_K$  as to produce a change in a positive direction. We find that in negative cells the change is always in a positive direction up to 150 mv.<sup>10</sup> In positive cells it is in a negative direction (up to 50 mv.).

*The Influence of OH<sup>-</sup> on the Total Potential and on the Potassium Effect.*—When a cell in contact with 0.01 M NaCl is exposed for a time to KOH (or in some cases to NaOH) and again placed in contact with 0.01 M NaCl it may be found that the potential has increased by an amount varying from 10 to 80 mv. An increase in the potassium effect is often observed after exposure to OH<sup>-</sup>. (The potassium effect is the change in a positive direction observed when 0.01 M KCl is replaced by 0.01 M NaCl.<sup>16</sup>)

<sup>14</sup> In many cases the process was reversible (see footnote 10).

<sup>15</sup> Regarding partition coefficients see Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312; Osterhout, W. J. V., *J. Gen. Physiol.*, 1939-40, **23**, 171; Some models of protoplasmic surfaces, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, **8**, 51.

<sup>16</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13**, 715; 1939-40, **23**, 429; 1944-45, **28**, 23.

## DISCUSSION

The relatively high mobility of  $\text{OH}^-$  in  $X$  in negative cells is very striking. We find that in such cells the apparent value of  $v_{\text{OH}} \div u_{\text{Na}}$  is 4.2 (p. 46) and the value of  $v_{\text{OH}} \div u_{\text{K}}$  is 7.8 (p. 47).

This might be explained on the ground that  $X$  contains a weak organic acid, possibly a fatty acid, which tends to raise the value of  $v_{\text{OH}}$ , perhaps in the same way that guaiacol raises the mobility of certain ions in  $X$ .<sup>17</sup> Furthermore, if an acid  $\text{HA}$  is present so that KOH entering  $X$  reacts to form  $\text{KA}$  and if  $v_A$  has a high value this would have the same effect as if  $v_{\text{OH}}$  had a high value: hence the apparent high value of  $v_{\text{OH}}$  may include the value of  $v_A$ , as already stated (p. 44). This would imply that  $v_A > u_{\text{K}}, u_{\text{Na}}$  but this might be explained on the ground that in place of  $\text{K}^+$  and  $\text{Na}^+$  in  $X$  we have complex ions of the type  $\text{KX}_I$ ,  $\text{KX}_{II}$ , etc., as assumed by Kraus and by Fuoss.<sup>18</sup>

We find that on standing in KOH the apparent value of  $v_{\text{OH}}$  falls off (p. 51); this does not happen to the same extent with NaOH (p. 49). This suggests an analogy with ordinary soaps where the fatty acids are more rapidly washed out by KOH than by NaOH. On this basis we might expect the fatty acid to dissolve out and lower the apparent value of  $v_{\text{OH}}$  much more rapidly in KOH than in NaOH.

If  $X$  contains an acid it is not surprising that the partition coefficients for NaOH and for KOH appear to be high (p. 53). An analogy is found, for example, when  $\text{Ba}(\text{OH})_2$  is shaken with isoamyl alcohol and only a small amount of barium enters but when 0.01 M oleic acid is added to the isoamyl alcohol a relatively large amount of barium is taken up, as shown in a previous paper.<sup>19</sup> In our calculations we have considered only the partition coefficients of NaOH and KOH but it is evident that we have to do also with  $\text{NaA}$  and  $\text{KA}$  if an acid,  $\text{HA}$ , is present in  $X$ .

It has been found necessary to assume the presence of an acid in  $X$  to account for the rate of entrance of  $\text{NH}_3^{20}$  and of guanidine<sup>21</sup> into *Valonia*.

Furthermore, the fact that basic dyes readily penetrate  $X$  while acid dyes do not may be taken to indicate the acid nature of  $X$ .

The presence of a fatty acid in  $X$  might explain the increased resistance observed by Blinks<sup>22</sup> in *Valonia* after the application of acetic acid. A model

<sup>17</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1939-40, **23**, 171, 749.

<sup>18</sup> Cf. Kraus, C. A., *Tr. Electrochem. Soc.*, 1934, **66**, 179. Fuoss, R. M., *Chem. Rev.*, 1935, **17**, 27.

<sup>19</sup> Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, **21**, 553 (footnote 29).

<sup>20</sup> Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1935, **21**, 125.

<sup>21</sup> Jacques, A. G., *Proc. Nat. Acad. Sc.*, 1935, **21**, 488.

<sup>22</sup> Blinks, L. R., The relations of bioelectric phenomena to ionic permeability and to metabolism in large plant cells, in *Cold Spring Harbor symposia on quantitative biology*, Cold Spring Harbor, Long Island Biological Association, 1940, **8**, 204.

of this has been constructed by applying acetic acid to a layer of soap which sets free oleic acid and thus increases the resistance.<sup>23</sup>

The fact that KOH is more effective than NaOH in changing the behavior of the protoplasm falls in line with other observations. Cells live longer<sup>24</sup> in 0.01 M NaCl than in 0.01 M KCl.

Returning now to Fig. 4 it may be said that it shows a general resemblance to the curves produced by other reagents in *Valonia* and in *Nitella*. But these do not seem to depend on the same factors as the curve in Fig. 4. For example, the application of guaiacol to *Valonia* causes the curve to fall and then rise, and leaves the cell in an altered state.<sup>25</sup> But guaiacol does not produce such effects in *Nitella*.<sup>26</sup> Somewhat similar curves are produced in *Valonia* by aniline,<sup>27</sup> benzene,<sup>28</sup> nitrobenzene,<sup>28</sup> and hexylresorcinol.<sup>29</sup> The curves in Figs. 3 and 4 resemble somewhat those obtained by applying KCl plus formaldehyde<sup>16</sup> or plus mercuric chloride<sup>24</sup> where the value of  $P_x$  (potential at X) at the start is equal to that of  $P_y$  (potential at Y, the inner protoplasmic surface) and  $P_x$  falls off at the start more rapidly than  $P_y$ . Since this is not the case with Figs. 3 and 4 of this paper the resemblance cannot be regarded as significant. In all these cases the underlying causes may be quite different from those involved in Figs. 3 and 4 of this paper.

It might be suggested that the change from negative to positive cells under the influence of OH<sup>-</sup> is due to a shift whereby an amphotelyte in X passes to the opposite side of the isolectric point. Whether this is the case must remain undecided for the present.

One of the most interesting effects of KOH is the change from  $v_{OH} > u_K$ ,  $u_{Na} > u_K > v_{OH} > u_{Na}$  which, as already stated (p. 52), shows that in this case the protoplasmic surface is not a pore structure. It has been found that in *Valonia*<sup>30</sup> and in *Halicystis*<sup>31</sup> the normal order is  $u_K > v_{Cl} > u_{Na}$  and this can be changed to  $u_{Na} > v_{Cl} > u_K$  in *Valonia*<sup>32</sup> by guaiacol.<sup>33</sup>

The observations reported in this paper indicate that the protoplasmic surface in *Nitella* is acid in nature and that it is not a pore system.

<sup>23</sup> Osterhout, W. J. V., Some models of protoplasmic surfaces, in Cold Spring Harbor<sup>r</sup> symposia on quantitative biology, Cold Spring Harbor, Long Island Biological<sup>1</sup> Association, 1940, 8, 51.

<sup>24</sup> Unpublished results.

<sup>25</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, 20, 13, 685.

<sup>26</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, 22, 417; 1939-40, 23, 171, 749.

<sup>27</sup> Osterhout, W. J. V., *J. Cell. and Comp. Physiol.*, 1941, 18, 129.

<sup>28</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1940-41, 24, 699.

<sup>29</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1940-41, 24, 311.

<sup>30</sup> Damon, E. B., *J. Gen. Physiol.*, 1938-39, 22, 819.

<sup>31</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1939-40, 23, 53.

<sup>32</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, 20, 13.

<sup>33</sup> Guaiacol can change  $v_{Cl} > u_{Na}$  to  $u_{Na} > v_{Cl}$  in *Halicystis* (Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, 21, 707).

I wish to thank Mr. Harry Bodner for the care and skill he has shown in carrying out these experiments.

#### SUMMARY

Remarkable changes are brought about by KOH in transforming negative cells of *Nitella* (showing dilute solution negative with KOH) to positive cells (showing dilute solution positive with KOH). NaOH is less effective as a transforming agent.

This might be explained on the ground that the protoplasm contains an acid (possibly a fatty acid) which makes the cell negative and which is dissolved out more rapidly by KOH than by NaOH, as happens with the fatty acids in ordinary soaps.

Part of a negative cell can be changed to positive by exposure to KOH while the untreated portion remains negative.

After exposure to KOH the potential the protoplasm has when in contact with NaCl may increase. At the same time there may be an increase in the potassium effect; *i.e.*, in the change of P.D. in a positive direction observed when 0.01 M KCl is replaced by 0.01 M NaCl.

In some cases the order of ionic mobilities is  $u_K > v_{OH} > u_{Na}$ . This shows that the protoplasmic surface cannot be a pore system: for in such a system all cations must have greater mobilities than all anions or *vice versa*.

# THE ACCELERATION OF SAPONIN HEMOLYSIS BY PROFLAVINE

By H. BRUCE COLLIER

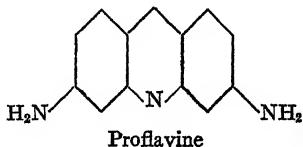
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## PLATE 1

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The observation that derivatives of phenothiazine accelerate hemolysis of horse erythrocytes by saponin and by lysolecithin (Collier and Allen, 1942 b) led to the suggestion that the acridine antiseptics, such as acriflavine and proflavine, might show similar properties. There are very few references in the literature to the effect of these compounds upon erythrocytes. Fleming (1917) reported an agglutination of human red cells by acriflavine, and Bohland (1919) claimed that intravenous injection of trypaflavine brought about a reduction in the erythrocyte count. Meleney and Zau (1925) found that injection of acriflavine into rabbits had no effect upon the red cell count or fragility; *in vitro*, however, a marked increase in hypotonic fragility was produced.

The present investigation demonstrates that proflavine powerfully accelerates hemolysis of rabbit, human, and dog cells by saponin. Hemolysis by lysolecithin is, on the other hand, slightly inhibited. It was not possible to investigate the effect on oleate or bile salt hemolysis, as these lysins formed precipitates with the drug.



### Methods

The acridine antiseptics used were proflavine B.D.H.<sup>1</sup> (3,6-diaminoacridine sulfate, *Chem. Abstr.* system) and euflavine B.D.H.<sup>1</sup> (neutral acriflavine, a partially *N*-methylated diaminoacridine). In preliminary experiments these two drugs appeared to give identical results; consequently all subsequent experiments were carried out with proflavine alone.

The accelerating or inhibiting effect of these compounds on hemolysis was measured by the method of Ponder (1934) which involves the construction of time-dilution curves, and the determination of *R*, the ratio of dilutions giving the same degree of hemolysis in treated and untreated samples. The

<sup>1</sup> British Drug Houses.

*R* values reported below were determined from 10 minute dilutions in all cases, but were found to be virtually constant at various intervals up to 10 minutes.

The actual conditions for hemolysis and method of measurement were those described by Wilbur and Collier (1943). Erythrocytes from defibrinated blood were washed three times with buffered saline, pH 7.4, and made up to 1:250 dilution in the same saline. Hemolysis was followed photoelectrically, at a temperature of 22–23° C., and 50 per cent hemolysis was taken as the end-point.<sup>2</sup>

#### RESULTS

*Rabbit Erythrocytes*.—Time-dilution curves were obtained with suspensions of rabbit erythrocytes in buffered saline and in the saline containing proflavine at a concentration of  $2 \times 10^{-4}$  M (about 1:15,000), the cell suspension being added to the proflavine solution immediately before the determination.

The results of a typical experiment are illustrated in Text-fig. 1, from which it is seen that saponin hemolysis is very strongly accelerated, whereas the action of lyssolecithin is slightly inhibited. (Under the conditions of the experiment proflavine alone, in the absence of lysin, caused no hemolysis or shape change in the cells.) In experiments on blood from four animals the average *R* value for saponin hemolysis was  $0.48 \pm 0.01$ . The millimolar acceleration constant,  $A = \frac{1-R}{c}$ , (Ponder and Hyman, 1939) is therefore about 2.6.

The *R* value for lyssolecithin hemolysis was  $1.11 \pm 0.03$ .

In order to make comparison with a substance of known accelerating action a parallel experiment was carried out with indole (Eimer and Amend) at  $10^{-3}$  M concentration. The values thus obtained were:

$$\text{Saponin, } R = 0.85; A = 0.15$$

$$\text{Lyssolecithin, } R = 0.90; A = 0.10.$$

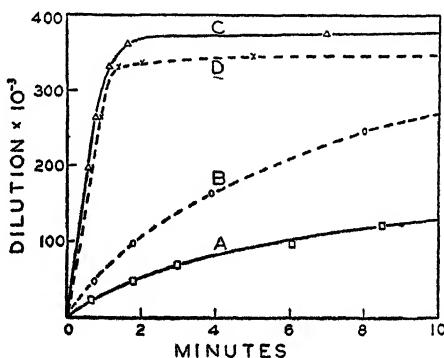
Ponder (1941) using saponin and indole obtained an *R* value of 0.32; his determinations were, however, carried out at 25° C. in unbuffered saline.

*Human Erythrocytes*.—Cells from two specimens of human blood were tested as described above, with essentially similar results. The proflavine alone caused no hemolysis or shape changes. For saponin the *R* values were 0.62, 0.63;  $A = 1.9$ . Lyssolecithin was slightly inhibited ( $R = 1.05$ ), the degree of inhibition being almost within the limits of experimental error.

<sup>2</sup> The photoelectric opacity method for measuring per cent lysis of a cell suspension has been justified by a comparison of opacities with cell counts, using very slow hemolysis by saponin and by lyssolecithin. The two methods agreed within the limits of experimental error. Centrifugation and determination of the Hb concentration of the supernatant gave results that indicated too high a degree of hemolysis.

*Dog Erythrocytes.*—When saponin was added to a suspension of dog cells in  $2 \times 10^{-4}$  M proflavine it was observed that the opacity of the suspension at first increased and then decreased as hemolysis set in. This alteration in opacity indicated a change in shape of the erythrocytes, which was confirmed by microscopic observation.<sup>3</sup>

The changes in dog erythrocytes caused by proflavine are illustrated in Fig. 1. The washed cells in buffered saline (glass slide and cover-slip) were crenated spheroids with a diameter of about  $6 \mu$  (Fig. 1 a). Five minutes after the addition of proflavine the forms seen in Fig. 1 b were observed. Many were V-shaped or X-shaped; others resembled masses of crystals. Of especial interest are transitional "hat-shaped" forms with a hemispherical



TEXT-FIG. 1. Effect of proflavine,  $2 \times 10^{-4}$  M, on hemolysis of rabbit erythrocytes. Curve A, saponin, standard curve; curve B, saponin + proflavine; curve C, lysolecithin, standard curve; curve D, lysolecithin + proflavine.

outline upon a rim. After 30 minutes all the cells had changed to rods (Fig. 1 c) of about  $13 \mu$  in length and  $2 \mu$  in width.

The deformation was apparently not due to a reaction with the cell contents. In the presence of saponin similar changes occurred, but loss of hemoglobin took place, finally revealing only the faint outline of rod-shaped ghosts. (As mentioned above, rabbit and human erythrocytes remained unaltered in shape in the presence of proflavine.) Because of these changes in form and in opacity

<sup>3</sup>The measurement of opacity of a cell suspension in the photoelectric photometer is a sensitive means of detecting shape changes in the erythrocytes. For example, addition of saponin to a suspension of rabbit, human, or dog cells gives rise to a transitory increase in opacity suggesting disking (Wilbur and Collier, 1943), the washed cells being usually crenated spheroids. Under the microscope it was confirmed that addition of saponin brought about an initial flattening prior to hemolysis. Sodium oleate causes this flattening to a marked degree, while lysolecithin causes immediate spherization.

it was impossible to measure precisely the accelerating action of proflavine on saponin hemolysis of dog erythrocytes. In two experiments apparent *R* values of 0.71 and 0.85 were obtained; the actual acceleration would be greater than is represented by these values.

#### DISCUSSION

It has been found that proflavine is a very powerful accelerator of saponin hemolysis of the erythrocytes of the rabbit, man, and dog. The acceleration constant of 2.6 for rabbit cells is of the same order of magnitude as was obtained by Ponder (1939) for the halogen derivatives of benzene and naphthalene, and is greater than the constant, 0.7, for indole (Ponder, 1941).

Lysolecithin hemolysis is not accelerated by proflavine but is actually inhibited to a slight degree. This fact appears to offer further evidence that saponin and lysolecithin have different mechanisms of lytic action (Wilbur and Collier, 1943). As suggested by Ponder (1941, 1943) the accelerating substance almost certainly acts through its effect upon the erythrocyte membrane. This alteration in the cell membrane (possibly a stretching or compression of the film, or a change in the surface forces, as indicated by shape changes) may facilitate or retard the action of a given lysin, depending upon its mode of action.

A possible explanation of the action of the acridine derivatives is that they combine with cephalin, which is a part of the erythrocyte membrane (see Ponder, 1943). Chargaff and Ziff (1939) observed that cephalin forms insoluble compounds with certain basic dyes; and we have found that proflavine forms precipitates with both cephalin and lecithin.

No explanation can be offered for the peculiar shape changes observed in dog erythrocytes in the presence of proflavine. The rod-like forms resemble those observed by Florman and Wintrobe (1938) in cases of ovalocytosis. Diggs and Bibb (1939) have described cells found in cases of sickle cell anemia which are somewhat like the proflavine forms.

It may be of interest to note that the thiazine derivatives and the acridine derivatives appear to have the following properties in common: antibacterial action; inhibition of cholinesterase (Collier and Allen, 1942 *a*); acceleration of hemolysis (Collier and Allen, 1942 *b*).

*Addendum.*—In preliminary experiments with the new antiseptic, 9-aminoacridine, supplied through the kindness of Dr. G. J. Martin, Research Director of the National Drug Co., Philadelphia, the following results were obtained:

There were no shape changes in dog, rabbit, or human erythrocytes. In hemolysis experiments at  $2 \times 10^{-4}$  M concentration the *R* values were: saponin—rabbit cells 0.56, dog cells 0.64; lysolecithin—rabbit cells 1.09, dog cells 1.08. The hypotonic fragility of rabbit erythrocytes was very slightly increased by the same concentration of the drug.

## SUMMARY

Proflavine is a very powerful accelerator of saponin hemolysis of rabbit, human, and dog erythrocytes. Lysolecithin hemolysis, on the other hand, is inhibited.

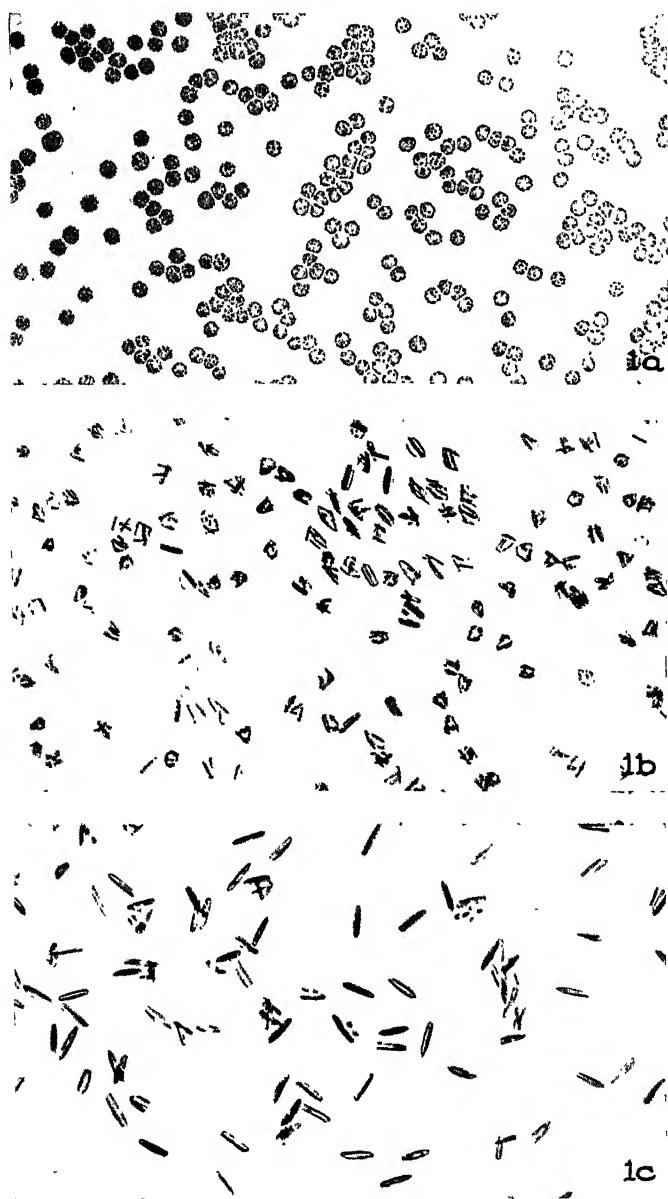
Dog erythrocytes in the presence of proflavine undergo marked changes in shape, finally becoming rods of about  $13 \mu$  in length. Rabbit and human erythrocytes are not altered in form under these conditions.

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## EXPLANATION OF PLATE 1

FIG. 1. Effect of proflavine on dog erythrocytes. (a) Untreated cells; (b) after 5 minutes; (c) after 30 minutes.  $\times 330$ .



(Collier: Acceleration of saponin hemolysis by proflavine)



'THE EFFECTS OF CAFFEINE ON OXYGEN CONSUMPTION AND  
CELL DIVISION IN THE FERTILIZED EGG OF THE SEA  
URCHIN, *ARBACIA PUNCTULATA*

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INTRODUCTION

As a phase of the general study of variation produced by caffeine upon the reproductive phenomena in animals, outlined by Cheney (1944), the inhibitory influence of this purine compound upon the oxidative processes associated with development is of interest. The data presented are intended to show the relative (not absolute) effects of several caffeine concentrations in sea water upon the relative  $O_2$  consumption values for fertilized *Arbacia* eggs and its correlation with the effect upon cleavage rate.

Progressive development following fertilization is determined by a chain of respiratory reactions, a system upon which cell division depends and the "activity" phase of which is responsible for a considerable (70 to 90 per cent) percentage of the total  $O_2$  consumption of the fertilized egg. The remainder (10 to 30 per cent) of the  $O_2$  consumption involves the "resting" or "basal" system. Ball and Meyerhof (1940) indicated the presence of an oxidative enzyme upon which respiratory inhibitors act in unfertilized *Arbacia* eggs. Krahl, Keltch, Neubeck, and Clowes (1941) reported a cytochrome oxidase enzyme, in the fertilized *Arbacia* egg, which can oxidize reduced cytochrome C. These authors demonstrated that this enzyme, when acting with cytochrome C as a substrate, is inhibited completely by sodium cyanide or sodium azide, just as these agents depress cell division in the fertilized living *Arbacia* egg. In contrast, the total respiration can be inhibited only to a maximum of 70 to 80 per cent by cyanide and only about 50 per cent by azide. Oxygen consumption is also inhibited by carbon monoxide and hydrogen sulfite. Data by Fisher, Henry, and Low (1944) indicate that the  $O_2$  inhibition effect (55 per cent) of sulfanilamide, resulting in complete suspension of cell division, is exerted on the main or activity system. They also state that the inhibition of  $O_2$  uptake seems to be the way in which this agent interferes with cell division in the fertilized sea urchin egg. These authors, in a study of the cyanide inhibition of  $O_2$  uptake by the unfertilized and fertilized sea urchin eggs, feel that it is difficult to escape the conclusion that fertilization introduces a respiratory system upon which cell division depends and which is responsible for 40 to 50 per cent of the  $O_2$  consumption of the fertilized egg. They assume apparently that the pathways of oxidation of the

substrate are different in unfertilized and fertilized eggs. Ball (1942), in discussing oxidative mechanisms in animal tissues, has pointed out that the evidence derived from cyanide inhibition of O<sub>2</sub> uptake in a fertilized egg and the absence of its inhibitory effect upon the unfertilized egg (*i.e.* upon fertilization the egg consumes O<sub>2</sub> at a greatly increased rate and the additional O<sub>2</sub> consumed is cyanide-sensitive) *merely indicates* the possible existence in the unfertilized eggs of a *system alternate* to the cytochrome system. Ball suggests that it is possible that a reaction can occur *directly* between cytochrome oxidase and some flavoprotein in the *Arbacia* egg. This may be true also in the mechanism of biological oxidations in the animal body in its general energy relationships. The chief process by which foodstuffs appear to be oxidized in the living cell has been schematized by Ball (1944) to indicate the relationship of the cytochrome oxidase enzyme to the process of respiratory metabolism as follows: foodstuff—H<sub>2</sub>—pyridine nucleotides—H<sub>2</sub>—flavoprotein—cytochrome B—C—A—cytochrome oxidase— $\frac{1}{2}$ O<sub>2</sub> + H<sub>2</sub> → H<sub>2</sub>O. This scheme applies also to the chain of reactions in the fertilized sea urchin egg metabolism in the experiments reported here. Caffeine would seem, by inference derived from the data in this paper, to act upon the activity phase of that system. The activity system normally accounts for the greater part of the total O<sub>2</sub> consumption by the fertilized sea urchin egg but this activity phase is inactive in the resting cell according to Fisher and Henry (1944).

The mechanism involved is of significance to all research on growing cell metabolism including postfertilization phenomena. Fertilized sea urchin eggs are excellent material for studies on the effect of a substance upon the respiration of a cell because they depend so completely upon O<sub>2</sub> uptake for the utilization of nutrients, since they possess no anaerobic metabolic activity. Caffeine has a stimulatory effect upon the O<sub>2</sub> consumption in some animal tissues. Saslow (1937) reported an increase in O<sub>2</sub> consumption in unstimulated caffeinized striated muscle of *Rana pipiens* Schreber. He cites the average O<sub>2</sub> consumption of muscles in Ringer solution of 32 mm.<sup>3</sup> per gm. (wet) per hour in contrast with the average O<sub>2</sub> consumption of muscles in 0.037 to 0.042 per cent caffeine-Ringer of 201 mm.<sup>3</sup> per gm. (wet) per hour. Saslow also states that no lactic acid accumulates in caffeinized muscles in oxygen. Wortis (1935), however, in the case of the brain tissue of the rat, presented evidence that caffeine sodio-benzoate in high concentrations (0.5 per cent) diminishes the O<sub>2</sub> consumption. Thus the literature indicates a distinct variation with regard to the effect of caffeine upon the O<sub>2</sub> uptake phase of the respiratory metabolism of animal cells. At least divergent results have been reported for different tissues. This situation is not necessarily disturbing in view of the fact that certain tissues, such as striated muscle, possess oxidation mechanisms upon which caffeine might act as in a trigger release fashion; whereas, the fertilized sea urchin egg has no such mechanism. In the current data presented here, caffeine in the higher concen-

trations has been found to be inhibitory on the  $O_2$  uptake by fertilized sea urchin eggs.

A fractionation of normal respiration into an activity and a resting portion might explain some cases of inhibitory action, *i.e.* one phase of the system might be more sensitive than the other to the agent used; or, lower concentrations of caffeine may merely be ineffective. For the sea urchin egg, Korr (1937) and Ballentine (1940) and, for the frog muscle, Stannard (1941) have reported fractionations of oxygen consumption similar to that described for narcotics. The relationship of caffeine inhibition of  $O_2$  consumption to the concentration of this agent is expressed by Fig. 2 which is derived from a typical experiment, No. 10, of the series. It will be noted later that the degree of inhibition of cell division with increasing concentrations of caffeine approximates this same curve.

#### *Materials and Experimental Method*

Eggs and sperm of *Arbacia punctulata* were collected as described by Just (1939), exercising care to avoid contamination with body fluids and to utilize only egg batches in which 95 per cent or better fertilization had occurred. This was determined by microscopic examination at 100  $\times$  for the presence of the fertilization membrane. The eggs were concentrated by low speed centrifugation and only 35 per cent suspensions in sea water or caffeine-in-sea-water were employed. A total volume of 2 cc. for each Warburg vessel was maintained. Each flask contained 0.5 cc. of the 35 per cent suspension of fertilized eggs, thereby assuring an equal number (volume) of eggs per vessel. The pure alkaloid, caffeine U.S.P. Merck, was used. The caffeine-in-sea-water concentrations ranged in percentages and approximate molarities as follows: 0.002 per cent ( $M/10,000$ ), 0.004 per cent ( $M/5,000$ ), 0.02 per cent ( $M/1,000$ ), 0.10 per cent ( $M/200$ ), 0.2 per cent ( $M/100$ ), 0.5 per cent ( $M/40$ ), 2.0 per cent ( $M/10$ ).

The manometric determinations of  $O_2$  consumption were made with the Warburg-Barcroft apparatus using the Warburg direct method technique described by Dixon (1934). All experiments were performed at 25°C. and a shaker rate of 83 oscillations per minute with an 8 cm. amplitude. The pH of the sea water in the laboratory system at Woods Hole during the period of these experiments was 7.94. The annual average pH for open sea water at Woods Hole is 8.2 (Harvey, 1932). The addition of caffeine resulted in making the caffeine-in-sea-water 0.05 more alkaline, pH 7.99. This alkalinity increase is not significant since Smith and Clowes (1924) showed that the cell division rate in *Arbacia* is quite independent of a pH range between 6.0 and 8.3.

Controls (in nearly all cases, duplicate controls were run and showed a difference of less than 2 per cent) and thermobarometric checks (blanks) were recorded for all readings. Results were observed at 15 minute intervals over a 3 hour period. At the termination of each experiment, cell division was stopped in all of the solutions by the addition of formalin to 1 cc. of the contents of each flask to make its concentration equivalent to 0.1 per cent in sea water. The remainder of the flask contents was examined also microscopically for the condition of the eggs without formalin. The

cleavage stage reached by the eggs in each suspension was compared with the controls. The rate of cell division is expressed as a percentage of the normal (control) rate.

#### EXPERIMENTAL RESULTS

The 15 experiments reported here showed an average oxygen consumption of 66 mm.<sup>3</sup> O<sub>2</sub> per hour for the normal, non-caffeine-treated, uninhibited, fertilized sea urchin egg respiration during the 3 hour duration of the experiments.<sup>1</sup> Plotting the data of the oxygen consumption of the controls and the experimental series of seven different concentrations of caffeine-in-sea-water as an O<sub>2</sub> consumption-time relationship curve (Fig. 1) reveals an increasing degree (average) of inhibition of O<sub>2</sub> consumption from 23.4 per cent with 0.1 per cent (M/200) to 61 per cent with 2 per cent (M/10) concentrations of caffeine.

TABLE I  
*Variable Data for O<sub>2</sub> Consumption by Fertilized Arbacia Eggs in Lower Caffeine Concentrations*

No. of experiment	Per cent concentration = Molarity	Average increase in O <sub>2</sub> uptake	Average decrease in O <sub>2</sub> uptake
10	per cent 0.02	per cent 0	per cent 10.6
5	"	" 6.2	0
7	0.004	M/5,000 0	6.2
8	"	" 6.1	0
12	0.002	M/10,000 0.44	0
3	"	" 0.70	0

The inhibition of O<sub>2</sub> uptake may be correlated with the effect on the cleavage rate or complete suppression of the cleavage process in the higher percentages. See Table II for the condition of the eggs at the termination of the 3 hour period in the Warburg flasks approximately 3½ hours after fertilization.

It may be observed from Fig. 3 that the degree of inhibition of the cleavage process approximately parallels the O<sub>2</sub> inhibition curve seen in Fig. 2. A distinct plateau showing a maximum depression of O<sub>2</sub> consumption and a complete inhibition of cell division is indicated by these curves in the instance of all caffeine percentages of 0.5 per cent or higher.

It will be noted in Fig. 1 that a progressive sequence of inhibition occurred in the experimental eggs from 0.1 to 2.0 per cent caffeine-in-sea-water inclusive. 2 per cent approaches the limits (2.2 per cent) of solubility of caffeine-in-sea-water. The effects noted in the remaining lower percentages were variable,—

<sup>1</sup> To prevent the possibility of mechanical cytolysis and to avoid the danger, emphasized by Whitaker (1933), of cortical injury which would abnormally increase the rate of respiration, no attempt was made to concentrate the eggs to a maximum.

see Table I. In five experiments the data for 0.02 per cent suggest stimulation instead of the inhibition indicated by the average of all data for 0.02 per cent in the series. This variability in the effect of lower percentages causes one to consider whether or not there is some sort of competitive action for control of the oxidative steps in the metabolism.

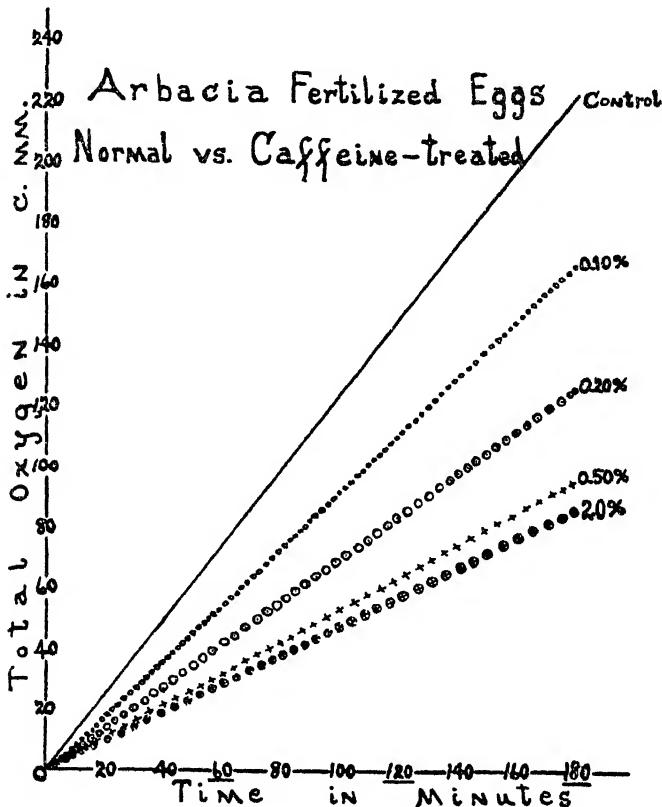


FIG. 1. O<sub>2</sub> consumption-time relationship graph.

However, changes of less than 10 per cent, whether inhibitory or stimulatory, were considered not significant in biological material of this sort. All the variable data occurred only in experiments involving a concentration of less than 0.10 per cent caffeine. The lower concentrations are either ineffective or possibly act upon a different phase of the respiratory metabolism. The writer is of the opinion, however, that the evidence indicates that the caffeine affects only a single site on the main pathway (activity phase).

Most inhibitors, sodium cyanide in particular, have their action upon the main pathway in cellular respiration. Cyanide probably has its action at the

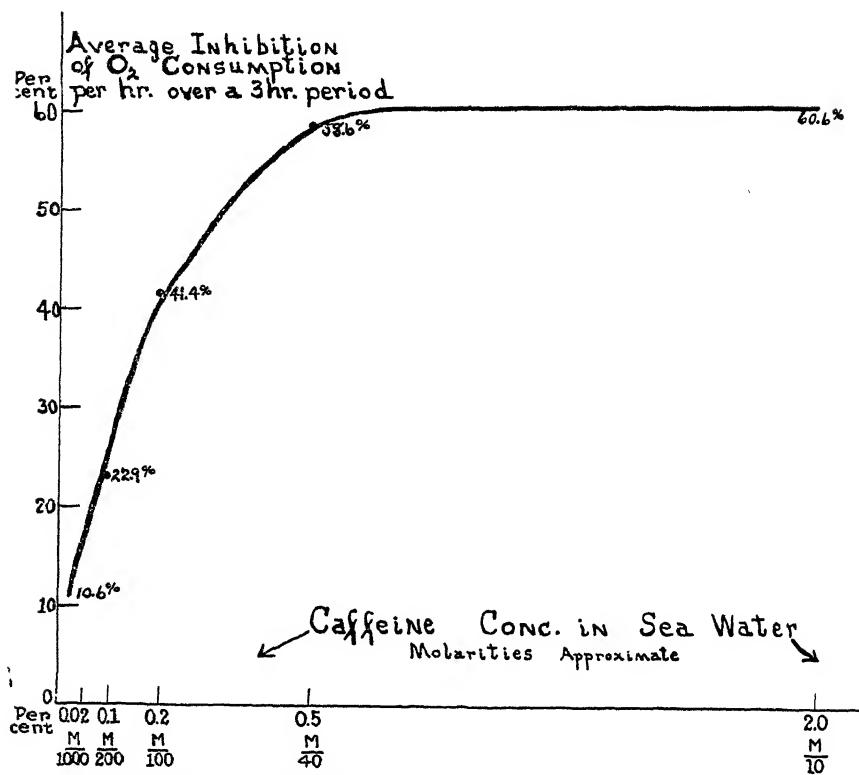


FIG. 2. Relationship of caffeine concentration to inhibition of O<sub>2</sub> consumption.

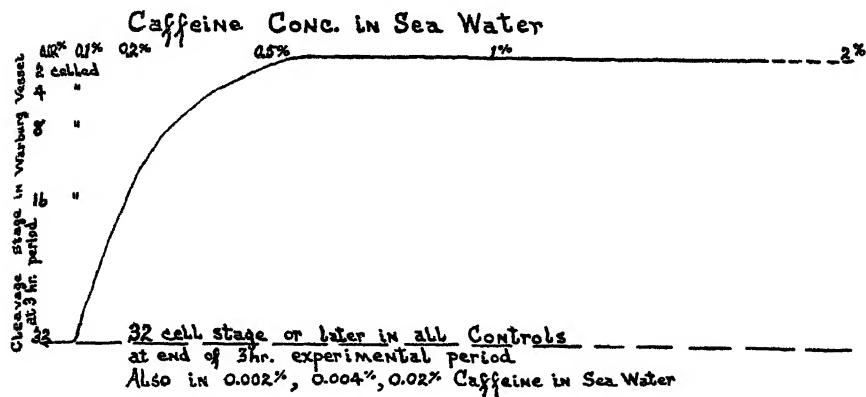


FIG. 3. Relationship of caffeine concentration to the degree of cleavage inhibition.

cytochrome oxidase point from the evidence of Krahl, Keltch, Neubeck, and Clowes (1941) and others. I have no direct evidence that caffeine acts specifi-

TABLE II

Composition of experimental medium	Egg suspension used 35 per cent in sea water	Caffeine-in-sea-water added	Sea water added	Alkali (10 per cent KOH) added to center well	Condition of eggs in Warburg vessel at end of experiment 3½ hrs. after fertilization
Control	cc.	cc.	cc.	cc.	Normal 64 and 32 celled late cleavage stages
0.002 per cent CSW*	0.5	0	1.3	0.2	Similar to controls
0.004 " " "	"	1.3	0	"	" " "
0.02 " " "	"	"	"	"	Mostly late cleavages. Few showed tendency to clump. Rarely red echinochrome pigment was clumped in egg
0.10 " " "	"	"	"	"	32 celled mostly. Few 64 celled. 5 per cent with tendency for pigment to clump. ±1 per cent with pigment actually clumped
0.20 " " "	"	"	"	"	16 celled stage. Some 8. Very few 32. 40 per cent with tendency to clump. Actual clumping in a few
0.50 " " "	"	"	"	"	No cleavage but 50 per cent "appeared" normal microscopically. In 45 per cent, tendency to clump. In 5 per cent, pigment was clumped
2.0 " " "	"	"	"	"	No cleavage. Complete suspension of division process. Red echinochrome concentrated in one area in 100 per cent of eggs, usually in center, sometimes eccentric but always localized

\* CSW = caffeine-in-sea-water.

cally on the cytochrome oxidase enzyme but it should be noted that the caffeine maximum inhibition of O<sub>2</sub> consumption is above 60 per cent and cyanide maximum inhibition is above 70 per cent. Caffeine and cyanide depression of O<sub>2</sub> uptake in fertilized sea urchin eggs is, therefore, of the same general order of magnitude.

Caffeine would appear to exert its influence then somewhere on the primary (activity) pathway, especially when it is noted that the cell division inhibition seems to parallel the  $O_2$  consumption depression. The  $O_2$  consumption is undoubtedly the major energy source for the cell division process. Cleavage stages were recorded in all controls and experimental vessels and compared with corresponding percentages in parallel experiments of the series. Table II shows that 2 per cent ( $M/10$ ) and 0.5 per cent ( $M/40$ ) caffeine-in-sea-water blocked cleavage completely. An examination of Fig. 1 indicates that the normal uptake, in comparison with the controls, was depressed over 50 per cent. Cleavage rate, however, is not necessarily limited by the rate of  $O_2$  consumption *per se* in a number of forms. For example, Amberson (1928) found the cleavage rate was not retarded seriously in *Arbacia* eggs, when  $O_2$  pressure was diminished, *until* the pressure reduction was sufficient to decrease the  $O_2$  consumption to about half normal. My findings agree with this generality in so far as cleavage was not completely inhibited otherwise.

#### SUMMARY

1. By means of the Warburg-Barcroft microrespirometer apparatus and the Warburg direct method, the relative effect of caffeine upon the  $O_2$  consumption of the fertilized egg of *Arbacia punctulata* was shown for the following concentrations in sea water: 0.002 per cent ( $M/10,000$ ), 0.004 per cent ( $M/5,000$ ), 0.02 per cent ( $M/1,000$ ), 0.1 per cent ( $M/200$ ), 0.2 per cent ( $M/100$ ), 0.5 per cent ( $M/40$ ), and 2 per cent ( $M/10$ ).
2. In comparison with the normal eggs (uninhibited, non-caffeine-treated controls), caffeine in concentrations including and greater than 0.1 per cent ( $M/200$ ) depressed the average uptake from approximately 25 to 61 per cent over the 3 hour period. In a number of instances, as typified by Experiment 10, the effective inhibitory concentration ranged from 0.02 per cent ( $M/1,000$ ) upward and the degree of depression of the  $O_2$  consumption ranged from 10.6 per cent to 60.6 per cent.
3. All caffeine concentrations including and above 0.02 per cent ( $M/1,000$ ) in the series used, resulted in decreasing the normal rate of cleavage division in the fertilized *Arbacia* eggs.
4. The higher concentrations (0.5 and 2 per cent) produced a complete blockage of the cleavage process.
5. Complete cleavage inhibition was noted only when the  $O_2$  uptake had been depressed to 50 per cent or more of the normal controls.
6.  $O_2$  consumption-time relationship data indicate an average depression, in  $O_2$  consumption over a 3 hour period, ranging from 25 per cent with a caffeine concentration of 0.1 per cent to a 61 per cent inhibition with a concentration of 2 per cent.
7. Concentrations of less than 0.1 per cent (certainly of less than 0.02 per cent) give variable results and indicate no significant effect.

8. It is inferred from the respiration data presented that it is probable that the inhibition of the O<sub>2</sub> consumption in fertilized *Arbacia* eggs is due to the influence of caffeine upon the main (activity or primary) pathway. It will be observed that there are certain similarities of the caffeine data to the degree of inhibition accomplished by sodium cyanide. Moreover, it has been demonstrated that the cyanide probably acts on the cytochrome oxidase step in the cytochrome oxidase-cytochrome chain of reactions constituting the O<sub>2</sub> uptake phase of respiratory metabolism. It is not improbable, therefore, that caffeine also may act upon the cytochrome oxidase enzyme.

9. From the viewpoint of environmental conditions influencing reproductive phenomena, it is of interest that caffeine can affect the normal metabolism of the zygote.

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## WATER RELATIONS IN THE CELL

### I. THE CHLOROPLASTS OF NITELLA AND OF SPIROGYRA

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(Received for publication, May 1, 1945)

Of fundamental importance in the metabolism of the cell is the behavior of water. Of especial interest is the giving up of water by one part of the cell to another as an indication of changes in metabolism or constitution.

An example of this is seen in the changes in volume of the nucleus which may occur under normal conditions in certain cells.

Such transfers can be produced in *Nitella* experimentally. When cells<sup>1</sup> are exposed to 0.5 M NaCl the salt may penetrate the outer protoplasmic surface more rapidly than the inner so that water is withdrawn from the sap and the protoplasm increases in volume at the expense of the vacuole.

Another example is seen in the decrease in volume often observed in the chloroplasts of *Nitella*<sup>2</sup> under natural conditions. This may also be produced experimentally.

The normal condition of the chloroplasts is shown in Fig. 1. They are imbedded in the aqueous layer *W* of the protoplasm lying between the outer, non-aqueous surface layer of the protoplasm *X*, and the corresponding inner layer, *Y*.<sup>3</sup> The outer portion of the layer *W* consists of a stiff gel containing the chloroplasts. The inner portion of *W* is liquid and is usually in active motion.<sup>4</sup> When sufficient centrifugal force is applied the long rows of chloroplasts may separate and each row then acts as if it were a stiff gel forming a long straight rod of protoplasm containing a single series of chloroplasts.

When cells, carefully removed from their natural surroundings, are brought into the laboratory and examined it is often possible to find areas where the chloroplasts have contracted to form spherical (or nearly spherical) bodies (Fig. 1). Since the diameter of the sphere is not greater than the smaller diameter of the ellipsoid body of the chloroplast before contracting it is evident that there must be a loss of volume and a consequent transfer of water to other parts of the cell. To what extent this must be attributed to injurious or patho-

<sup>1</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1943-44, 27, 139.

<sup>2</sup> The observations were made on *Nitella flexilis*, Ag. The cells were freed from neighboring cells and observed at once or kept in the laboratory in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, 17, 87) at 15°C. ±1°C. An hour before use the temperature was raised to about 25°C.

<sup>3</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1944-45, 28, 23.

<sup>4</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1944-45, 28, 17.

logical processes is an open question, as in the case of the contractions in *Spirogyra* to be described later.

Such contraction may also be brought about experimentally. For example, 0.01 M lead acetate produces it in a few minutes. After this the protoplasmic motion continues for 30 minutes or more. If the cell is then washed in water and left for 3 days we find that the turgidity is normal and when acid fuchsin is applied it does not penetrate sufficiently to color the sap although some of the large spherical bodies in the sap may take up some color (this also happens in normal cells not previously exposed to reagents). Evidently little or no injury has occurred. In some cases cells were kept for 7 days under a coverglass in a solution of 0.01 M lead acetate without loss of turgidity.

If as soon as the contraction occurs the cell is rinsed repeatedly in water and allowed to stand in water the chloroplast resumes its normal dimensions as an

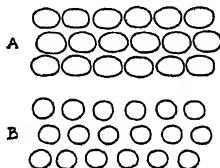


FIG. 1. *A*, chloroplasts of *Nostoc* in normal state. *B*, after contraction. Diagrammatic.

ellipsoid body in about 30 minutes. If the motion is stopped by the lead acetate it is resumed after washing the cell with water.

This operation, causing contraction and recovery, may be repeated 4 or 5 times on the same cell with intervals of 10 minutes in water between the exposures to lead acetate and the cell may recover completely from the last operation.

If in place of 0.01 M lead acetate we apply 0.01 M lead nitrate we find little or no contraction. This is perhaps less surprising in view of the fact that the two salts differ considerably in their chemical properties (e.g., in precipitating proteins).

Some other substances cause contraction before death ensues, but so far none have been found which give such striking results as lead acetate. Among these may be mentioned digitonin<sup>5</sup> (0.01 per cent) which may produce contraction in 6 minutes after which motion may continue for 20 minutes. In ferric chloride (0.01 M or 0.001 M) contraction may occur in 40 minutes and the motion may continue for a few minutes longer after which the cell dies.

A variety of substances cause contraction simultaneously with death, as shown by the rapid entrance of acid dyes, such as acid fuchsin and eosin.

<sup>5</sup> The effect does not appear to be due to an inorganic impurity since it disappears when the digitonin is ashed.

The chloroplast of *Spirogyra* may undergo remarkable contractions accompanied by loss of water. It may change from a long, spirally coiled ribbon to a short, nearly straight rod.

Cells may exhibit various stages of contraction in their natural environment out-of-doors, as stated by de Vries,<sup>6</sup> and more frequently when they are kept in the laboratory.

Contractions may also be produced experimentally. O. Loew<sup>7</sup> noticed that potassium oxalate caused a contraction of the edges of the chloroplast but did not observe that it produced any longitudinal contraction.

Unaware<sup>8</sup> of the observations of de Vries and of Loew, the writer treated *Spirogyra* with a variety of salts and obtained longitudinal contractions with BaCl<sub>2</sub> and SrCl<sub>2</sub><sup>9</sup> at low concentrations. Later, S. S. Chien,<sup>10</sup> working in the writer's laboratory, found that CeCl<sub>3</sub> was still more effective. In all these cases the contractions were caused without killing the cells.

Later, Scarth<sup>11</sup> made a careful study of such contractions, finding that they can be caused by a great variety of salts and that in general the effect tends to increase with the valency of the cation. He found that contractions are also produced by alcohol and acetone, rise of temperature, and electric shocks.

A species of *Spirogyra* which is very favorable for such studies has recently become available to the writer and it is upon this that the following observations are based.

These have been greatly aided by a device which enables us to keep the same individual cell under observation during changes of solutions. A sheaf containing 1 to 10 filaments was placed lengthwise along the center of a slide and held securely in place at each end by placing upon it a drop of liquid gelatin (20 per cent) warmed to 29°C. The gelatin quickly cooled, fastening the filaments in place and the temperature of 29°C. did not alter the cells except perhaps in some cases those very close to the gelatin. In some cases a third drop was placed midway between the other two, leaving room for a coverglass (3/4 inch square) on each side of the central drop of gelatin. This makes it possible to apply different solutions to the same filament on opposite sides of the central drop of gelatin.

<sup>6</sup> de Vries, H., *Ber. deutsch. bot. Ges.*, 1889, 7, 19.

<sup>7</sup> Loew, O., Physiological role of mineral nutrients, *U. S. Dept. Agric., Division Vegetable Physiol. and Path., Bull. 18*, Washington, 1899.

<sup>8</sup> For a knowledge of the observations of de Vries and of Loew the writer is indebted to the papers of Scarth.

<sup>9</sup> Osterhout, W. J. V., *Am. J. Bot.*, 1916, 9, 481.

<sup>10</sup> Chien, S. S., *Bot. Gaz.*, 1917, 63, 406. In Fig. 2 of this paper the words "large form, crassa type, pyrenoids omitted" should be transferred from Fig. 2 to Fig. 1. In the summary "CeCl<sub>3</sub>" should read "CaCl<sub>2</sub>".

<sup>11</sup> Scarth, G. W., *Tr. Roy. Soc. Canada, Section V*, 1922, 16, 51; 1923, 17, 137; *Quart. J. Exp. Physiol.*, 1924, 14, 99, 115.

Lines were scratched across the slide at intervals by means of a diamond and numbered to facilitate the location of individual cells.

The solutions in contact with the cells could be changed without removing the coverglass by placing filter paper at one side and adding solution at the other. To make sure of good irrigation a little dye may be added to each new solution.

The acid fuchsin and eosin were made by the National Aniline Co.

The results are very variable, depending on the season of the year, the period of standing in the laboratory, and the medium. In many cases distilled water proved a good medium in which to keep the cells in the laboratory, in other cases a saturated solution of  $\text{CaCO}_3$  or Solution A.<sup>12</sup>

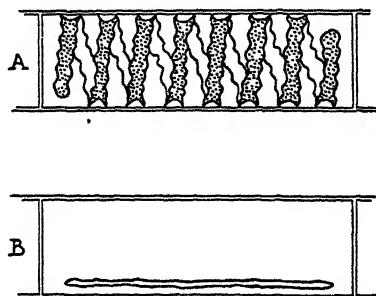


FIG. 2. *A*, cell of *Spirogyra* in normal state. *B*, after contraction of the chloroplast produced by 0.01 M  $\text{BaCl}_2$  (without plasmolysis). Pyrenoids omitted. Diagrammatic.

A normal cell is shown in Fig. 2 (in many cells the spiral is less loosely wound than in Fig. 2).<sup>13</sup> The diameter varies from 25 to 35 microns and the length from 200 to 300 microns. The thickness of the cell wall<sup>14</sup> is not over 3 microns.

In most of the filaments no nucleus could be demonstrated by killing with 0.33 M acetic acid and staining with eosin or with Heidenhain's iron hematoxylin. Photographs of these filaments by ultraviolet light (2537 Å.) failed to reveal a nucleus but showed good absorption by the chloroplast. I wish to thank Dr. G. I. Lavin for making these photographs.

In a few filaments, however, a nucleus, more or less typical, could be demonstrated. In other cases the nucleus may adhere to the chloroplast and thus be difficult to demonstrate.

The chloroplast appears bright by dark-field illumination and is more highly refractive than its immediate surroundings.

<sup>12</sup> Cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, 17, 87.

<sup>13</sup> Perhaps more than one species is present but if so their reactions were quite similar.

<sup>14</sup> The longitudinal cell wall is regarded as consisting of three layers, the two inner of cellulose and the outer of pectose: the end wall is regarded as pectose with a layer of cellulose on each side of it. Cf. Smith, G. M., *Fresh-water algae of the United States*, New York, McGraw-Hill Book Co., Inc., 1st edition, 1933, 554.

The chloroplast appears to lie between the outer (*X*) and inner (*Y*) surfaces of the protoplasm, as in *Nitella*. De Vries<sup>6</sup> locates it in this position on the basis of plasmolytic experiments. The fact that salts, such as BaCl<sub>2</sub>, do not produce contraction at once may indicate that time is required to penetrate and reach the chloroplast. The *X* and *Y* surfaces remain in place; no plasmolysis occurs.

The chloroplast is a clear, green ribbon spirally coiled. It has pyrenoids at regular intervals (these are not shown in the figure). The width of the ribbon varies greatly but does not, as a rule, exceed 10 microns. The edges may be smooth or greatly crenated or serrulated.

A convenient method of observing contraction is by means of BaCl<sub>2</sub>. When 0.01 M BaCl<sub>2</sub> is applied contraction may begin within a few minutes but the time varies greatly (in some cases no contraction occurs). The contraction may begin by a withdrawal of the ends of the chloroplast so that the distance between them and the end walls increases. The main contraction, however, consists in a shortening of each coil. This involves a loosening of the chloroplast from the protoplasm which remains in contact with the cell wall. This may continue until the chloroplast has become fairly straight (as in Fig. 2). When this is the case the length of the chloroplast may be reduced to 20 per cent or less of the normal and at the same time the width of the ribbon may decrease. Hence unless the thickness of the ribbon, measured in a direction perpendicular to the cellulose wall, has increased at least 5 times there must have been a loss of volume. In many cases it is easy to ascertain by focussing that no such increase in thickness has occurred so that we may say that there is a loss of volume. This means that water has been given up to the other parts of the cell. This applies to such cells as show contraction in their natural environment as well as to those in which it is produced by experimental treatment. This contraction occurs while the cell is still alive, as evidenced by plasmolysis and by the fact that acid fuchsin and eosin do not enter.

When the contraction is less extensive it may be difficult to determine whether a loss of volume has occurred. Scarth<sup>15</sup> states that it occurs in certain cells, especially under the influence of heat, acetone, and alcohol, but that in some cases the volume may remain constant or show a slight increase.

In our experiments there was no evidence of reversibility of the contraction when the cells were removed from the reagent and allowed to stand in water. Scarth speaks of reversibility in some cases but this required several days.

#### SUMMARY

Chloroplasts may contract under natural conditions and give up water to the rest of the cell, thus indicating changes in metabolism or constitution. Such contractions may be produced experimentally.

<sup>15</sup> Scarth, G. W., *Quart. J. Exp. Physiol.*, 1924, **14**, 107.

In *Nitella* the chloroplasts are ellipsoid bodies which, under natural conditions, may contract to spheres with a loss of volume. This may be brought about by lead acetate, ferric chloride, and digitonin: the contraction may occur while the cell is alive. The contraction in lead acetate is reversible (in lead nitrate little or no contraction occurs).

In *Spirogyra* the chloroplast is a long, spirally coiled ribbon which may contract under natural conditions to a short nearly straight rod with a loss of volume. This can be brought about by inorganic salts and in other ways while the cell is still alive.

# THE ACTION SPECTRUM OF SENSITIZATION TO HEAT WITH ULTRAVIOLET LIGHT\*

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Sensitization of organisms to heat by first irradiating them with ultraviolet light was described for paramecia by Bovie and Daland (1923), for bacteria by Curran and Evans (1938), and for yeast by Anderson and Duggar (1939, 1941). By sensitization is meant killing by a sublethal dosage of heat following a sublethal dosage of ultraviolet light. In these experiments the effects of heat and light are not merely additive since heating to the same extent before irradiating has no effect. An apparently analogous sensitization of proteins *in vitro* is observed after irradiation with ultraviolet light. Flocculation occurs in such solutions if the temperature is raised above 4° C., whereas unirradiated solutions remain clear (Clark, 1938). Since proteins are the main structural constituents of organisms, an explanation of light sensitization to heat as an effect upon the cellular proteins seems logical. However, Clark (1938) believes the evidence is inadequate to justify such a conclusion. More data are desirable to determine whether proteins or some other cellular constituents are involved. By determining the relative effectiveness of each of a number of ultraviolet wave lengths in sensitizing the organisms to heat, an action spectrum can be obtained which presumably represents the absorption spectrum of the class of chemicals responsible for sensitizing the organism to heat. The results for paramecia suggesting proteins in this rôle are described below.

## *Materials and Methods*

*Paramecium caudatum* and *Paramecium multimicronucleata* were used as experimental animals and were grown in mass cultures (Giese and Taylor, 1935) or in isolation culture (Giese, 1945a) as required. A quartz mercury arc served as a source and the light was passed through a natural quartz monochromator. The following wave lengths were used in the experiments: 2483, 2537, 2654, 2804, 3025, 3130, 3350, and 3660 Å. The intensity was determined with a thermopile calibrated against United States Bureau of Standards lamps. Dosages of 1000, 2000, 3000, and 4000 ergs/mm.<sup>2</sup> were generally used, but occasionally other dosages were used. About 50 to 100 paramecia were irradiated each time and a sample of these, held in a capillary pipette, was quickly injected into 5 cc. of pond water contained in each of a series of test tubes held in a constant temperature bath at 42.3°C. After a given exposure, timed with a

\* This work was supported in part by funds from The Rockefeller Foundation.

stop-watch, the paramecia were quickly poured into cool pond water contained in a large watch glass and observed with a dissecting microscope. The time required to immobilize a majority of the paramecia was taken as the lethal time. After preliminary trials the end point was determined with more precision and five repetitions were made. In this way repeatable results were obtained. While the lethal time is not always the same for the controls, the relation between the sample irradiated with a given dosage and the control from the same culture is fairly constant.

## EXPERIMENTAL

### 1. *The Action Spectrum*

Experiments on sensitization to heat by irradiation with ultraviolet light of both *P. caudatum* and *P. multimicronucleata* give essentially the same results. The data for both species are summarized in Table I and for *P. caudatum* in Figs. 1 and 2. A maximum effect is observed at the shortest wave length tried, 2483 Å. In fact, it is impossible to give a greater dosage than 1000 ergs/mm.<sup>2</sup> because in that case killing upon exposure to heat becomes so rapid that the measurements are unreliable. A smaller maximum is observed at 2804 Å. Between these two wave lengths lies a minimum. At wave lengths longer than 2804 Å the effectiveness declines rapidly falling to zero at 3660 Å.

For comparison, the absorption spectrum of pseudoglobulin is added to Fig. 2. The action spectrum for sensitization to heat resembles the absorption spectrum of pseudoglobulin. The data suggest that the simple proteins of the cytoplasm are sensitized to heat by the absorption of ultraviolet radiations. While the fit is imperfect both at the short and at the long wave length ends of the spectrum, no other substance in protoplasm, organic or inorganic, has selective absorption resembling the action spectrum even to this extent (for absorption spectra see Casperson, 1936).

All the experiments described involve irradiating the paramecia (UV) first, then heating them (H). The order is therefore (UV), (H). If the same exposures are given in the reverse order, namely (H), (UV), the paramecia are not killed. The experiment therefore describes a true sensitization, not an additive effect of two injurious agencies.

### 2. *Effects of Heat and Ultraviolet Light upon the Division Rate*

Since Anderson and Duggar (1941) found that yeast lost its ability to form colonies after an exposure to 2650 Å followed by heat, they concluded that the division mechanism was sensitized to heat by irradiation. Previous work on effects of ultraviolet light alone upon division rate indicates an action spectrum resembling nucleoprotein absorption (Giese, 1945 b). If ultraviolet light also sensitizes nucleoproteins to heat, an action spectrum for heat sensitization corresponding to absorption by nucleoproteins should also be found. While this is not the case for lethal dosages on paramecium as shown above, it might

TABLE I  
*Sensitization to Heat by Ultraviolet Radiations*

Wave length	Intensity	Ultraviolet dosage, ergs/mm. <sup>2</sup>				
		0	200	500	750	1000
		Relative heat exposure to kill				
<i>1. Paramecium caudatum</i>						
2483	2.0- 3.4	100	79	36	24	10
		Ultraviolet dosage, ergs/mm. <sup>2</sup>				
		0	1000	2000	3000	4000
		Relative heat exposure to kill				
2537	2.8-13.0	100	81	57	40	22
2654	4.4-11.1	100	89	53	33	17
2804	3.7- 7.1	100	69	46	21	9
3025	5.2-12.1	100	83	73	58	48
3130	15.3-16.2	100	89	76	65	55
3350	3.1	100	93	94	87	71
3660	30.2	100	—	—	—	96
<i>2. Paramecium multimicronucleata</i>						
2537	2.8-13.0	100	64	40	30	19
2654	4.4-11.1	100	84	49	30	18
2804	3.7- 7.1	100	72	47	20	11
3025	5.2-12.1	100	84	68	60	49
3130	15.3-16.2	100	92	83	74	59
3350	3.1	100	90	81	74	60
3660	30.2	100	—	—	—	90
		Ultraviolet dosage, ergs/mm. <sup>2</sup>				
		0	200	500	750	1000
		Relative heat exposure to kill				
2483	2.0- 3.4	100	64	25	22	13

At zero dosage the actual lethal exposure time is usually 6 to  $6\frac{1}{2}$  minutes. The widest range in time is  $5\frac{1}{2}$  to  $8\frac{1}{2}$ . For zero dosage 100 units equals 6 minutes in the first experiment cited, then 79 units equals  $4\frac{3}{4}$  minutes. As dosage increases the relative exposure needed to kill decreases.

hold for sublethal effects. Therefore, the division rate of paramecia exposed to sublethal doses of ultraviolet light followed by sublethal doses of heat was studied. Dosages of 750 ergs/mm.<sup>2</sup>, or less, of ultraviolet light and 3/4 or less

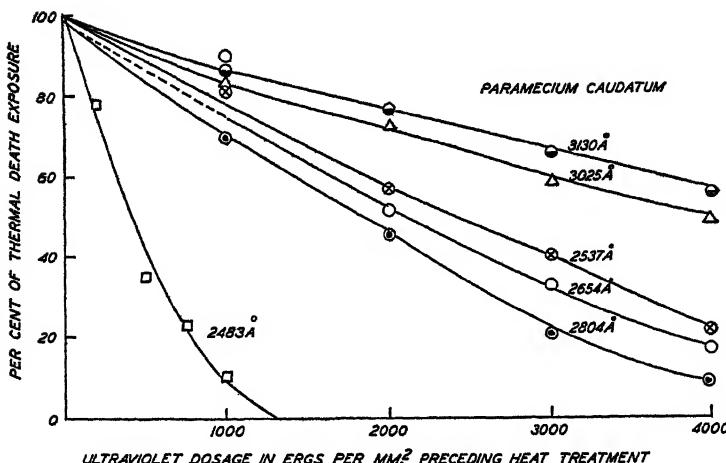


FIG. 1. Relative effectiveness of various wave lengths in sensitizing paramecia to heat. All the curves are smooth except the one for 2654 Å. The point at 1000 ergs/mm.<sup>2</sup> lies above all the others. In all five experiments with *P. caudatum* and five with *P. multimicronucleata* the same result was obtained. The significance of this deviation is not understood.

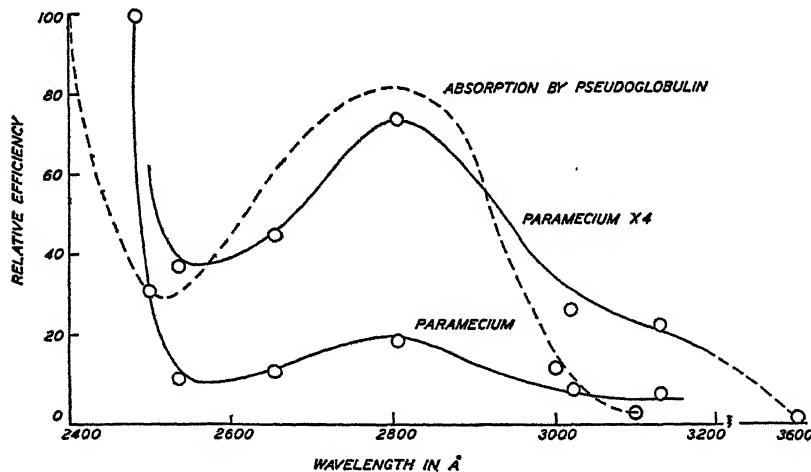


FIG. 2. Action spectrum for sensitization to heat. Pseudoglobulin absorption after Smith (1929), nucleic acid absorption after Casperson (1936). The values for 3000 ergs/mm.<sup>2</sup> were used for all wave lengths except 2483 for which 750 ergs/mm.<sup>2</sup> was used. In paramecium X 4 to show more clearly the 2800 Å maximum, the data were multiplied by 4 for all wave lengths except 2483 Å.

of the lethal thermal time were employed. Such paramecia were compared with those merely irradiated on the one hand and those merely heated to the same extent on the other. Such exposure to heat alone has no effect on the division rate other than causing a slight lag before division begins. Such exposure to ultraviolet light alone reduces the rate of division in addition to the lag (see Giese, 1939). Heat after ultraviolet radiation has no effect at all or merely increases the lag phase slightly. The experiments under the conditions tried therefore do not offer any evidence of sensitization of the division mechanism to heat by irradiation with ultraviolet light. However, what seems to be

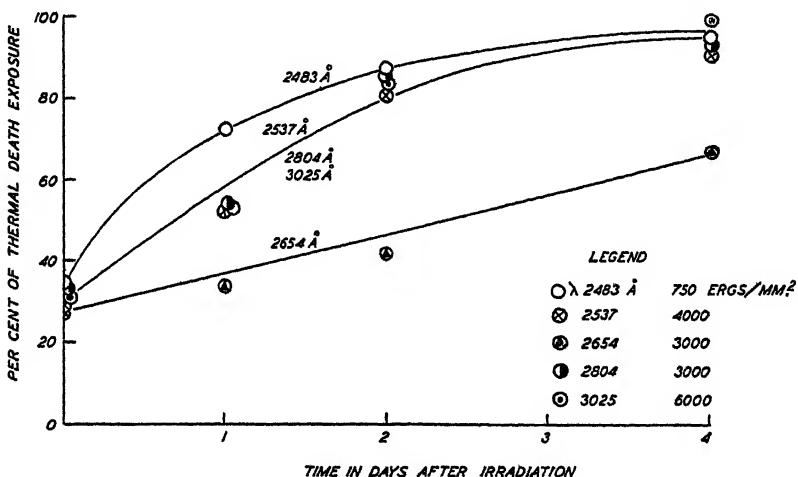


FIG. 3. Recovery from heat sensitization at different wave lengths. The same or about the same initial sensitization values are taken as the basis of comparison for different wave lengths. The paramecia undergo division during this recovery period.

an effect on the nucleoproteins is demonstrable in the studies on the recovery of paramecia described below.

### *3. Recovery from Effects of Irradiation*

By irradiating the paramecia on one day and subsequently determining the lethal thermal dosage after various intervals of time the rate of recovery from the radiations could be determined by their loss of sensitivity to heat. To compare the rate of recovery at different wave lengths paramecia were given dosages which produce about the same degree of sensitization to heat and the recovery from such dosages is given in Fig. 3 and Table II. If the same material is being affected by each of these wave lengths the recovery from a comparable initial effect should be the same at each of these wave lengths. This

is true for all of the wave lengths tested except 2654 Å which indicates that more than one material is being affected. Since 2654 Å is absorbed especially strongly by the nucleoproteins, the finding suggests that nucleoproteins may be involved in heat sensitization. The results are interesting in another way—they show how long the effects of ultraviolet light, even in sublethal dosages, persist in the protoplasm of the cell.

TABLE II  
*Recovery from Sensitization to Heat Resulting from Ultraviolet Radiations*

Wave length Å	Dosage* ergs/mm. <sup>2</sup>	Lethal time after indicated number of days subsequent to irradiation			
		0 sec.	1 day sec.	2 days sec.	4 days sec.
2483	750	151	326	404	438
		122	277	322	399
	0‡	441	447	457	482
		465	459	483	454
2537	4000	130	242	364	428
	0	441	458	448	482
2654	3000	108	127	157	251
	0	390	370	412	370
2804	3000	155	241	400	427
	0	441	447	458	457
3025	6000	130	250	393	450
	0	436	451	458	450

\* Dosages to produce approximately the same initial retarding effect were selected.

‡ Zero dosage is the control in each case.

#### DISCUSSION

The similarity between the ultraviolet action spectrum of heat sensitization and the absorption spectrum of pseudoglobulin suggests that the substance in the cell affected by ultraviolet light and sensitized to heat is a simple protein, similar in its absorption spectrum to pseudoglobulin. However, *in vitro* experiments with another simple protein, albumin, show differences in responses to ultraviolet light and heat from those observed in living systems. Thus organisms are not sensitized by heating before exposure to ultraviolet light,

whereas according to Clark (1938) albumin shows a more copious precipitate if so treated. Furthermore, at any temperature above 4° C. the proteins will develop opacity due to aggregation of the albumin molecules denatured by the ultraviolet radiations, the rate increasing with the temperature, whereas in our experiments the organism shows no injury at temperatures below lethal. Thus paramecia subjected to sublethal dosages of ultraviolet light but kept at 33° C. continue to grow, at a retarded rate compared with unirradiated controls, but more rapidly than irradiated paramecia at 26° C. Even as the growth-inhibitory range for *Paramecium multimicronucleata* between 34.5° and 36.5° C. is approached there is no marked difference between unirradiated and irradiated specimens. It is only when temperatures lethal to the controls are approached that the phenomenon of sensitization in irradiated organisms is observed. Furthermore, even at the lethal temperatures sublethal exposure times have little or no effect on the paramecia, division of animals so treated after irradiation is not more retarded than division of animals irradiated but not heated.

Irradiation of pure protein solutions "sensitizes" them to heat in so far as a flocculum can be obtained even at low temperatures provided the temperature is above 4° C. (Clark, 1938). The term "sensitization" has little meaning in this case since the heat merely furnishes the energy for the secondary reactions involved in the formation of a flocculum. For a given dosage only a given amount of flocculum forms, the rate at which it forms is determined by the temperature. In the biological system on the other hand, no lethal effect is observed until the temperature approaches the thermal lethal threshold. In this case the heat does not merely furnish the energy for the secondary reactions involved in flocculation, since culture temperatures should suffice for that. The biological phenomenon therefore remains unique and certainly worthy of further analysis.

In contrast to biological systems proteins do not develop opacity at temperatures of 4° C. even if the dosage with ultraviolet radiations is sufficient to denature, as judged by heating a sample above 4° C., yet paramecia will vesiculate when irradiated even at 4° C. according to Clark (1938). As a result of this experiment Clark concluded that such organisms are not killed as a result of denaturation of the proteins, but by some effects on the membrane. The question may well be raised whether paramecia are dying because they are vesiculating or vesiculating because they have been injured internally and are dying. Evidence of specific action of ultraviolet light upon the cell membrane is lacking except for regions of the ultraviolet shorter than 2654 Å where activation of unfertilized sea urchin egg occurs (Hollaender, 1938). If the membrane is affected by longer wave lengths one might expect to find changes in permeability. Reed (1944 b) studying the effects of ultraviolet radiations on sea urchin eggs and Giese and Parpart (1940, unpublished) in similar studies on red blood cells found no evidence for changes in permeability to water and ethyl-

ene glycol even after dosages as large or larger than those used to sensitize in the present experiments. Reed (1944 a) has described a unilateral effect of ultraviolet light in which membrane formation is suppressed and the egg takes on a hemispherical shape. But this effect he explains as due to changes in viscosity in the egg rather than changes in the membrane. It is known that vesiculation of cells occurs as the result of almost any noxious agents slowly applied and that death from ultraviolet light or heat may occur without vesiculation. The vesiculation may result from the release of osmotically active materials inside the cell which enable it to take up more water, or in the case of paramecia to the paralysis of the contractile vacuoles (in this regard see Tschakotine, 1935). *Paramecium caudatum* pumps its own volume of water every 15 to 20 minutes (Adolph, 1931), therefore when water continues to come in through the membrane and fails to go out of the contractile vacuole cytolysis is bound to occur.

The data on recovery of paramecia from sensitization to heat after irradiation suggest that the nucleoproteins are involved since recovery from irradiation with one of the wave lengths absorbed most by nucleoproteins is slowest. The immediate effect of the ultraviolet radiation is apparently chiefly upon the general cytoplasmic mass of the cell, consisting mainly of simple proteins, and while the nucleoproteins are also affected they are present in too small a proportion to alter the general effect of sensitization to heat. Only when the cytoplasm recovers from the injury does the nucleoprotein effect become apparent, probably because recovery from nucleoprotein injury is slower. In this respect the data resemble the effects of ultraviolet on division of cells, in which the recovery action spectrum resembles nucleoprotein absorption (Giese, 1945 b). The action of ultraviolet light in sensitization to heat thus also appears to be multiple, rather than specific. Several constituents of the cell may be affected at the same time. The nature of the action spectrum will depend upon the reaction which is predominant or the criterion being used to judge the effects of the radiations. To get a true picture it is necessary to use as many criteria as possible.

#### SUMMARY

1. Heat does not sensitize paramecia to ultraviolet light but ultraviolet light sensitizes them to heat. Paramecia of two species (*Paramecium caudatum* and *P. multimicronucleata*) are much more readily killed by heat at 42.3° C. if they are first exposed to ultraviolet light.
2. From studies on paramecia irradiated with a given dosage at various wave lengths before being killed by heat, an action spectrum of the compound in the protoplasm being sensitized to heat can be determined. Proteins with absorption similar to that of pseudoglobulin are suggested by these experiments.

3. The effect upon living things differs from that on pure protein systems in that paramecia are not rendered more sensitive to temperatures below the lethal temperature whereas proteins are.
4. Almost complete recovery from ultraviolet light as judged by heat sensitivity occurs within 4 to 5 days.
5. By a study of the rate of recovery from doses at different wave lengths evidence suggesting effects on nucleic acid is obtained.
6. The possible significance of the data and the action spectrum is discussed.

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# THE PARACRYSTALLINE STATE OF THE RAT RED CELL

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In an addendum to a paper concerned with the relation between crenation and gelation (Ponder, 1944), a spontaneous change which takes place in rat red cells suspended in cold isotonic sodium citrate was described. As a result of this change the osmotic resistance of the cells becomes so increased that they do not hemolyze even in distilled water, and the cells otherwise behave as if they were gelated or paracrystalline. This paper is concerned with a more detailed investigation of the phenomenon, under four heads: (1) the resistance to osmotic hemolysis and its reversibility, (2) the exchange of K between the cells and the surrounding media, (3) the resistance to lysins such as saponin and digitonin, and (4) the shape transformations, the form changes observed during hemolysis, and the appearance under polarized light.

## 1. Resistance to Osmotic Hemolysis

The increase in resistance to osmotic hemolysis which the rat red cells develop can be followed by preparing a suspension of thrice washed red cells in 3 per cent sodium citrate, keeping it at 4°C., and adding one volume of the suspension to 9 volumes of water at various temperatures after various times from that of the making of the suspension up to several days.

The cells of 5 ml. of rat blood, received into 3 per cent sodium citrate, are washed several times with the same medium and are finally suspended in 25 ml. of the citrate; this gives a suspension with a volume concentration of about 0.1. The suspension is kept at 4°C.<sup>1</sup> and is shaken up occasionally, although the resuspension of the sedimented cells leads to a progressively increasing hemolysis.<sup>2</sup> From time to time, 0.5

<sup>1</sup> The rate at which the rat red cells in 3 per cent citrate become paracrystalline, as judged by the rate at which they become resistant to hemolysis by water at 4°C., depends on the temperature at which the suspension is kept. At 4°C., the change in state occurs within 48 hours; at 7°C., it takes about 96 hours; and at 10°C. it takes about 200 hours. The concentration of citrate in which the change of state occurs is not very critical.

<sup>2</sup> The cold rat red cells cannot be centrifuged and washed in the same way as normal red cells because they stick to each other when spun down; violent shaking is required to resuspend them, and this results in considerable hemolysis. The best way to keep the suspension in the refrigerator is in Erlenmeyer flasks, in which the cells sediment to form a loosely packed layer. Washing can then be carried out by sucking off the

ml. of the suspension is added to (1) 4.5 ml. of 3 per cent sodium citrate, and (2) 4.5 ml. of distilled water, the mixtures being maintained at various temperatures (4°, 20°, and 37°C.). After 10 minutes, which is sufficient time for the systems to arrive at their equilibria, the mixtures are centrifuged rapidly and the supernatant fluids are removed. The hemoglobin contents of each is found photometrically, and expressed as a percentage of the complete hemolysis of the systems.

The determinations of osmotic resistance are repeated at 12 to 24 hour intervals for several days, and Table I shows a series of typical results obtained at 4°, 20°, and 37°C. The table contains three columns for each temperature, the first (*S*) showing the percentage hemolysis observed in 3 per cent sodium citrate, the second (*W*) the percentage hemolysis observed in the hypotonic system (with *T* = 0.1, very nearly) in which the red cells are added to distilled water,

TABLE I

Time hrs.	4°C.			20°C.			37°C.		
	<i>S</i>	<i>W</i>	<i>h</i>	<i>S</i>	<i>W</i>	<i>h</i>	<i>S</i>	<i>W</i>	<i>h</i>
0	0	100	1.00	0	100	1.00	0	100	1.00
24	5	50	0.47	7	70	0.68	8	100	1.00
48	9	25	0.17	11	50	0.44	12	100	1.00
72	16	23	0.08	18	43	0.30	23	96	0.95
120	27	30	0.04	30	51	0.30	42	92	0.86

and the third the value of  $h = (W - S)/(100 - S)$ , which measures the hemolytic effect produced by the medium of tonicity 0.1. When there is complete hemolysis,  $h = 1.00$ ; when the water produces no more lysis than occurs in 3 per cent citrate,  $h = 0.00$ .

Table I shows (1) that the amount of lysis which takes place in the hypotonic system with *T* = 0.1 decreases with the time during which the red cells are kept at 4°C. in 3 per cent sodium citrate, becoming very small (*h* = 0.08) at the end of 72 hours when the measurements are made at 4°C., and considerably reduced (*h* = 0.30) at the end of the same time when the measurements are made at 20°C. This means that 92 per cent and 70 per cent of the red cells which do not hemolyze in citrate become resistant to osmotic hemolysis in this very hypotonic system at 4° and 20°C. respectively. (2) When the measurements are made at 37°C., the amount of lysis which occurs in the hypotonic system, in excess of that observed in isotonic citrate, becomes less as the time of preservation at 4°C. increases, but the great majority of the red cells undergo hemolysis at this temperature. (3) The longer the red cells are kept at 4°C., the more hemolysis is observed when they are added to isotonic sodium citrate

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supernatant fluid and replacing it with fresh citrate, and an estimation of the hemoglobin in the supernatant fluid provides a measure of the amount of spontaneous hemolysis.

at the various temperatures; thus 42 per cent of red cells kept for 5 days at 4°C. undergo hemolysis in 3 per cent citrate at 37°C. It is this spontaneous hemolysis which limits the duration of the experiments and gives rise to a variety of experimental difficulties.<sup>3</sup>

The state which results in the increase in resistance to hypotonic hemolysis is reversible in that it can be abolished by raising the temperature to 37°C. and reestablished by cooling to 4°C. This can be shown by an experiment of the following type.

A suspension of washed rat red cells in citrate is kept for 72 hours in the refrigerator at 4°C. The supernatant fluid is removed and replaced with cold 3 per cent citrate in order to reduce the amount of hemoglobin resulting from spontaneous hemolysis; the cells are then suspended by gentle shaking. To each of two tubes containing (a) 4.5 ml. of 3 per cent citrate at 4°C. and (b) 4.5 ml. of water at 4°C. is added 0.5 ml. of the suspension of the cold rat red cells. After 10 minutes the intact cells are thrown down, the supernatant fluid removed, and the percentage hemolysis determined from the hemoglobin contents (Table II, A).

The rat red cell suspension is now warmed to 37°C., and 0.5 ml. is added to (a) 4.5 ml. of citrate at 37°C. and (b) 4.5 ml. of water at 37°C., as before. At the end of 10 minutes the intact cells are spun down, and the percentage hemolysis determined (Table II, B).

The warmed suspension is then cooled to 4°C., and after 10 minutes 0.5 ml. is added to (a) 4.5 ml. of citrate at 4°C. and (b) 4.5 ml. of water at 4°C. After 10 minutes the intact cells are thrown down and the percentage hemolysis determined as before (Table II, C).

The results of an experiment of this kind are shown in Table II. At each temperature, the first column (*S*) gives the percentage hemolysis observed in 3 per cent citrate, the second column (*W*) that observed in the hypotonic system resulting from the addition of the suspension to water, while the third gives the value of *h*.

The warming of the cold rat red cells to 37°C. results in their becoming completely hemolyzable in a hypotonic system of *T* = 0.1; *i.e.*, they behave in the same general way as normal rat red cells do, except that the hemolysis which occurs in 3 per cent citrate is much greater (48 per cent in this experiment, but there is a great deal of variation; in Table I, for example, the corresponding figure is only 23 per cent). The lysis which occurs in 3 per cent citrate at 4°C.

<sup>3</sup> If 0.5 ml. of the cold rat red cell suspension is added to 4.5 ml. of 1 per cent NaCl instead of to the same amount of 3 per cent citrate, there is more hemolysis than in the citrate; the figures corresponding to those in the column for *S* at 4°C. in Table I, for example, would be 0, 8, 12, 23, and 50 per cent. In some experiments, indeed, more hemolysis was observed when the cold rat red cells were added to 1 per cent NaCl than when they were added to water. The cold rat cells seem to be less stable in NaCl than in citrate.

is also greater (17 per cent) than that which would occur with normal rat erythrocytes, and this is a sign that the cells are more unstable than normal rat red cells. The warming to 37°C. and the subsequent cooling apparently result in an increase in this instability, as is shown by the 40 per cent lysis in citrate in part C of Table II as compared with the 17 per cent lysis in part A of the same table.

Measurements of the change in volume which the paracrystalline rat red cells undergo in the hypotonic system of  $T = 0.1$  show that there usually is so little swelling as to be within the experimental error of the method used (high speed hematocrite with Hamburger tubes). The values obtained varied between 0.15 and zero. The variation in  $R$  may correspond, of course, either to a variation in the completeness with which the new paracrystalline state is reached, or to some of the cells having reached it completely while others have not.<sup>4</sup>

TABLE II

Treatment of cell suspension	<i>S</i>	<i>W</i>	<i>h</i>
A. 72 hrs. at 4°C.; added to citrate and water at 4°C.	17	37	0.24
B. Warmed to 37°C.; added to citrate and water at 37°C.	48	100	1.00
C. Cooled to 4°C.; added to citrate and water at 4°C.	40	53	0.22

### 2. Exchange of K

The exchange of K between cells and isotonic citrate can be measured by adding washed rat red cells to 3 per cent sodium citrate and adjusting the volume concentration to  $\rho = 0.1$  as determined by a high speed hematocrite. At intervals of 12 to 24 hours, the K content is determined both for the cells and for the supernatant fluid, the suspension being kept at 4°C. In the case of the determination of the K content of the cells, a small sample (2 ml.) of the suspension is removed after the contents of

<sup>4</sup> Dr. A. K. Parpart has kindly made measurements, by his opacimeter method, of the rates of penetration of water and of glycerol into rat red cells, washed and kept in 3 per cent sodium citrate at 9°C. for 9 days, and then allowed to stand for 2 hours at 25°C., the temperature at which the measurements were made. The principal results obtained are as follows: (a). In the cold rat red cell, and as judged by hemolysis, the penetration of glycerol is as rapid as, or more rapid than, the penetration of water. (b). As judged by hemolysis, water penetration is much slower (about 100-fold) in the paracrystalline cell than in the normal rat red cell. (c). As measured by volume changes with no hemolysis, the rate of water exosmosis in the paracrystalline cell is not very different from the normal rate, while the glycerol rate is less than normal. (d). The total volume change of the paracrystalline rat red cell in a glycerol-salt mixture of tonicity 2.0 is less (approximately one-fifth) than normal.

the container have been shaken up, and after (a) the volume concentration of the intact cells, and (b) the percentage number of cells which have hemolyzed spontaneously, have been determined. The 2 ml. sample is centrifuged and the supernatant fluid is removed as completely as possible. As the cells form a compact mass, the removal can be rendered all the more complete by allowing the tube to drain upside down, and by drying off the inside with filter paper. The K content of the cell mass, the volume of which is known, and also the K content of the supernatant fluid, are determined by the method of Breh and Gaebler (1930). Typical results are shown in the upper part of Table III.<sup>5</sup>

The K exchange which occurs when cold rat red cells, after some days in isotonic citrate at 4°C., are added to distilled water (*cf.* Tables I and II) can be measured by adding 1 ml. of the red cell suspension, the volume concentration of which is first determined, to 9 ml. of water at 4°C. After 5 minutes the unhemolyzed cells are thrown

TABLE III

	Time hrs.	K, cells <i>m.eq./l.</i>	K, fluid <i>m.eq./l.</i>
Cells in isotonic citrate at 4°C. $\rho = 0.1$	0	118.0	4.3
	18	—	6.7
	42	—	7.9
	72	67.5	11.3*
K exchange: Loss by cells = 50.5 m.eq./l.; gain by fluid = 53.5 m.eq./l.			
Cells, 2 ml., added to 18 ml. water at 4°C.	0.1 2	47.5 48.0	1.8 2.0†

\* 1.03 m.eq./l. due to hemolysis.

† 40 per cent lysis. Quantity calculated 1.8 instead of 2.0 m.eq./l.

down by centrifuging and the supernatant fluid is removed. This can be done very completely, since the cells form a sticky mass which can even be rinsed with a little cold water to remove traces of supernatant fluid. The K content of the cell mass, the volume of which is known, and also the K content of the supernatant fluid, are then determined. Photometric measurement of the quantity of hemoglobin in the supernatant fluid enables one to compute the percentage of the total number of cells which hemolyze in the hypotonic medium. Similar determinations of the K content of cells and supernatant fluid can be made after the cells have stood in the hypotonic

<sup>5</sup> The accuracy with which the values for K can be obtained largely depends on the accuracy with which  $\rho$  can be measured. This is not very great, and experimental errors of the order of 5 to 10 per cent are quite possible. It should be pointed out that in experiments of this kind, as well as in those on which Tables I and II are based, there are considerable variations which may be due to the extent to which the cells of the suspension have reached the final paracrystalline state. The results in Table III are representative of those of a number of experiments.

medium at 4°C., or at some other temperature, for times longer than 5 minutes. Typical results are given in the lower part of Table III.

Several points emerge from the data in Table III. (1) There is a considerable loss of K from rat red cells into 3 per cent sodium citrate, 43 per cent of the cell K being lost in 72 hours, and there is a corresponding gain of K by the surrounding medium in addition to the K which is gained because of progressive hemolysis. (2) When the cold rat red cells, with a K content of 67.5 m.eq./l., are added to a large volume of cold distilled water, about 30 per cent of their contained K is lost into the water, in addition to that which is lost as a result of the hemolysis of about 40 per cent of the cells. The remaining 60 per cent of the cells then remain unhemolyzed, although the intracellular concentration of K is some 25 times greater than that in the surrounding water. The extent to which the cells swell in the hypotonic medium is very small ( $R$  less than 0.15). (3) Most of the loss of K into the water probably occurs as soon as the cells are added to it, for the loss is substantially the same after 2 hours as it is after 5 minutes.

These results show that the failure of the paracrystalline rat red cells to swell and to hemolyze in water is not due to their reaching equilibrium with their hypotonic environment by losing so much K that the concentrations become equal inside and out. The two remaining explanations are (*a*) that the membrane of the cold rat red cell is impermeable to water, and (*b*) that the contained K is combined in some way as to make its activity approach zero, so that the driving force for the movement of water from a hypotonic environment approaches zero. Of these two alternatives, the latter is the more in keeping with other observations on the properties of the paracrystalline red cells.

### *3. Hemolysis by Saponin and Digitonin*

After standing for 72 hours in sodium citrate at 4°C., the cold rat red cells are considerably more resistant to the hemolytic action of saponin than are normal rat red cells when the measurements of the rate of hemolysis are made at low temperatures. The end-points, however, become steadily worse the longer the cells are allowed to stand in citrate at 4°C., so that ultimately it becomes impossible to measure the times for complete hemolysis in the ordinary way at a temperature as low as 4°C. The poorness of the end-point is due to the ghosts and debris of ghosts which can be seen by microscopic examination of the hemolyzed suspension; these disappear when the suspension is warmed.

To overcome the difficulty in determining the end-point for 100 per cent hemolysis, a time-dilution curve for a normal rat red cell suspension is plotted at 4°C., and then, after the cells have stood in citrate at a low temperature for several days, determinations are made of the percentage of complete hemolysis which results from allowing

the cold rat red cells to remain in contact with each dilution of lysin at 4°C. for the same length of time as is necessary for the complete hemolysis of normal rat red cells. The time-dilution curve for saponin and normal rat red cells at 4°C. is atypical,  $t$  passing through a minimum corresponding to a dilution of 1 in 1000 ( $2000\gamma/2$  ml.); the values of  $t$  for the normal rat red cells, and the percentage lysis  $P$  for the cold rat red cells after a time of contact  $t$  with the corresponding quantity of lysin, are shown in Table IV.

Except for the value corresponding to 16,000  $\gamma$ , the values of  $P$  are quite constant, which means that the various quantities of lysin produce about 32 per cent hemolysis of the cold rat red cell suspension in the same time as they produce 100 per cent hemolysis of the normal rat red cell suspension; *i.e.*, that the cold (paracrystalline) rat red cells are considerably more resistant to saponin at 4°C. The extent to which the resistance is increased cannot be calculated from the data, for the form of the resistance distribution is not known; if we

TABLE IV

$\delta, 1 \text{ in}$	$\gamma$	$t$	$P$
125	16,000	5.0	40
250	8,000	3.5	34
500	4,000	2.0	33
1,000	2,000	1.3	29
2,000	1,000	2.2	31
4,000	500	2.7	31
8,000	250	5.5	34
16,000	125	20	32

suppose it to have the usual negative skewness, however, the resistance of the paracrystalline rat red cells would be between 4 and 5 times that of the normal cells.

In the case of digitonin the situation is much the same as in the case of saponin. At 4°C. the end-points are poor even with normal rat red cells, and after 72 to 96 hours in cold citrate not more than 10 to 20 per cent hemolysis is produced by the same quantities of lysin and in the same times in which complete lysis of normal rat red cells is produced. The resistance of a suspension of cold rat red cells to digitonin is somewhere in the neighborhood of 8 to 10 times that of a suspension of normal rat red cells.

When the measurement of the times for complete hemolysis is made at 37°C., normal rat red cells and rat red cells which have been kept at 4°C. in citrate for 72 hours give almost identical time-dilution curves in systems containing either saponin or digitonin.<sup>6</sup> The quantity of lysin required to produce such

<sup>6</sup> Suspensions of cold rat red cells appear to be more resistant to digitonin at 37°C., but this can be shown to be due to the presence of substances, inhibitory for digitonin,

alterations in the membrane, the fixed framework, and the hemoglobin of the cells as are necessary to enable the hemoglobin to diffuse out freely into the surrounding medium is accordingly some 4 to 5 times greater when the cell is in its paracrystalline form than when the cell is a normal disk, and when the paracrystalline form is reconverted into the normal discoidal form by warming, the quantity of lysin required to bring about the conditions for hemolysis again becomes the same as that for normal rat red cells.

#### 4. Shape Transformations

When washed three times in 3 per cent sodium citrate, rat red cells appear as perfect biconcave disks between *plastic* slides and coverglasses. Between *glass* surfaces they undergo the usual disk-sphere transformation, but the spheres retain very fine crenations instead of becoming perfectly smooth. It is quite noticeable that their transformation is slower and less complete than it is in the case of human red cells in 1 per cent NaCl. On the addition of lecithin sols or saponin, the cells exhibit the usual shape changes.

After 72 hours in the refrigerator at 4°C., and when examined at 20°C., the cells appear as irregularly crenated biconcave disks, the edges of which are wrinkled or even look as if pieces had been cut out of them. They tend to clump, but there is little real rouleau formation when rat plasma is added. As the time during which they are kept at 4°C. increases, an increasing percentage of these cells show no shape changes between glass surfaces, or on the addition of lecithin, saponin, or rose bengal. After 3 days at 4°C., for example, about 50 per cent of the cells are able to undergo a more or less complete disk-sphere transformation between glass surfaces, while about 50 per cent remain discoidal. Again, after 7 days at 4°C., about 30 per cent of the cells hemolyze as perfect spheres on the addition of saponin, whereas the remainder lose their hemoglobin without any preliminary shape change.

The difference between the behavior towards lysins of normal red cells and that of rat red cells which have been kept for several days in isotonic citrate at 4°C. can most easily be appreciated by enclosing a drop of cell suspension between plastic surfaces, adding an approximately equal volume of 1 in 10,000 saponin, and observing the changes as they occur at room temperature. (1)

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in the medium in which the cells are suspended. For the amount of inhibitory substance present in 0.4 ml. of supernatant fluid,  $c_1/c_2 = 2$ , approximately. After the effect of this inhibition has been allowed for, the resistance of the cells themselves works out as substantially the same as that of normal rat red cells at 37°C. Some of the increased resistance of suspensions of cold rat red cells to digitonin at 4°C. may also be due to the presence of these inhibitory substances, in which case the true resistance of the cold rat red cells to digitonin at 4°C. would be about 4 or 5 times that of normal cells at the same temperature, instead of 8 to 10 times.

The majority of the cells kept at 4°C. do not undergo a disk-sphere transformation before they hemolyze, whereas normal rat red cells do. The majority of the cold cells retain their roughly discoidal biconcave form, and hemolyze as disks. (2) For a variable length of time before lysis occurs, the cold rat red cells develop a granular appearance in the neighborhood of the rim.<sup>7</sup> This does not occur in normal rat red cells, although lysis by saponin may be preceded by crenation. (3) When the cold rat red cells hemolyze, a granular debris is left behind; this is not seen after the lysis of normal rat red cells. The debris is observed particularly in the region which corresponds to the rim of the cell before its hemolysis. (4) Any prominent crenations or irregularities which are seen in the cold rat red cell before hemolysis persist in the hemolyzing ghosts. At times one even gets the impression that one part of the cell hemolyzes before the remainder, or that pigment is retained longer in the granular regions of the rim than in neighboring regions.

The cold rat red cells, when examined at 4°C. with polarized light, are birefringent, whereas normal rat red cells are not. The whole of the cold rat red cell is diffusely bright between crossed Nicols, the rim in general, and scattered areas in the rim in particular, being brightest of all. When the cold rat red cells are warmed to 37°C. for 10 to 20 minutes and then examined at room temperature with polarized light, the diffuse brightness of the cell is very much less, and the birefringence is confined to small irregular areas, usually in the region of the rim. These observations, for which I am indebted to Dr. Otto Glaser, are similar to the observations made by Teitel-Bernard (1932) on the formation of intracellular crystalline hemoglobin, and are compatible with the passage of hemoglobin, in the cold, into a crystalline or paracrystalline state. When the cells are warmed, most of the hemoglobin can be thought of as returning towards the less orderly state in which it exists in the normal red cell, loci of greater orderliness, however, remaining.<sup>8</sup>

#### DISCUSSION

The peculiarities in the behavior of the cold rat red cells can be accounted for by supposing that the cell components undergo a change of state from that

<sup>7</sup> This granular appearance occurs in the cells which have been kept in citrate in the cold, although not to the same extent as it does after the addition of a lysin such as saponin. The appearance of the rim is reminiscent of the "holly wreath" forms of the sickle cell, and the granular areas probably are regions in which the change in the direction of the paracrystalline state has proceeded further than elsewhere.

<sup>8</sup> There is a noticeable difference in color between a suspension of normal rat red cells and one of paracrystalline rat red cells. The difference is difficult to describe, as it consists in a difference of the shade of red, but it can easily be appreciated by anyone working with the material.

which characterizes the normal discoidal red cell to a state which can be described in the meantime as paracrystalline. The change of state is slow, its rate dependent on temperature, and it is probably not all-or-none with respect to any single cell, so that in the case of an assemblage of cells one cannot distinguish between the effects of different degrees of change of state and the involvement of different numbers of cells.

The evidence for the change of state being one in the direction of greater orderliness and crystal formation rests on the results of the observations with polarized light, on the inability of the cold rat red cells to undergo the usual shape transformations and to become spheres before hemolyzing, and on the apparent rigidity of the cells and of the ghosts. As the time during which they are kept at low temperatures increases, the cells show a steady increase in their resistance to lysis by hypotonic media, although they retain a large proportion of their electrolyte, and this can be interpreted either as showing that the cells become increasingly impermeable to water, or, more probably, that the activity of the originally osmotically active substances becomes less and less; in the individual cell, indeed, it probably becomes zero as the new paracrystalline state is ultimately reached. This state, however, is at least partially reversible by heating, and the familiar property of swelling and hemolyzing in hypotonic media reappears as the warmed cell passes towards the metastable state which characterizes the normal disk.

The ease with which this change of state occurs in the rat red cell may be related to the possibility, suggested by Drabkin (1945), that the hemoglobin of the rat erythrocyte is in an unusual metastable state approaching that of incipient crystallization, but the observations have more general implications. In the examination of the behavior of the mammalian red cell in hypotonic media, all varieties of volume increases have been observed, from those corresponding to  $R$ -values of 1.0 to those corresponding to  $R$ -values of about 0.5 (summarized by Ponder, 1940); the former are those which would be expected if the volume changes in hypotonic solutions were those of a "perfect osmometer" and brought about by the exchange of water alone, while the latter are very much smaller. Attempts have been made to account for the unexpectedly small volume changes on the basis of there being bound water in the cells or loss of osmotically active substances into the hypotonic environment, but while both these explanations go some distance towards accounting for the discrepancies between the observed volumes and those predicted on a simplified osmotic theory, neither accounts for them entirely or even largely. A different kind of explanation, which retains the idea of the red cell's being essentially a simple osmometer, accounts for the unduly small volume changes as resulting from there being an elastic resistance to swelling, such as there is in gelatin gels (Ponder, 1940, 1944). It is very doubtful whether the introduction of this

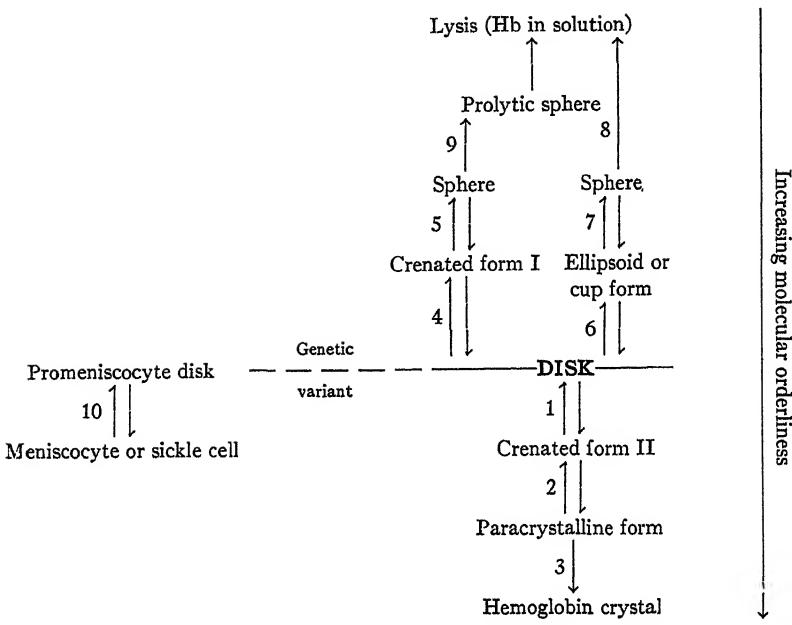
elastic resistance is anything more than another way of reconciling the observed results with the "expected" ones, but the idea has some connection with reality in that it arose from the observation that crenated red cells, which are cells in which the hemoglobin is gelated or paracrystalline, have  $R$ -values lower than those for red cells which are not crenated. Now that all  $R$ -values, from 1.0 to zero, have been obtained experimentally, it seems likely that  $R$  is nothing but an expression of the extent of the departure of the behavior of the real red cell, as regards volume changes, from that of the conceptual red cell of simplified osmotic theory. If the mammalian red cell can be represented as a simple osmotic system when  $R$  approaches 1.0, the smaller values of  $R$  would measure the extent to which the behavior of the simple osmometer is modified by the condition of the interior, either because the interior offers a resistance to swelling, or because the forces which tend to produce volume increases are smaller than those implied by the assumption of ideal behavior. One would anticipate, indeed, that in a system as condensed as the red cell interior the activities would be less than those in ideal solutions.<sup>9</sup>

The most interesting point about the change of state from that which characterizes the normal disk to that of the paracrystalline cold rat red cell is that it is reversible, so that the normal disk and the paracrystalline form can be looked upon as two metastable forms of the same cell. The molecular arrangements in the paracrystalline cold rat red cell are apparently more orderly than those in the normal disk, although not as orderly as those in the hemoglobin crystal, while the arrangements in the normal disk are themselves more orderly than when hemoglobin is in solution. In considering the behavior of the erythrocyte in media of varying tonicity and electrolyte content it has been customary to approach the problem from the standpoint of what would occur in dilute solutions separated by a membrane, *i.e.* from a very much simplified osmotic standpoint, but since the normal disk is a metastable form which passes spontaneously and reversibly into another metastable form which is paracrystalline, it is just as valid, and may be more profitable, to approach the problem of volume changes, etc., from the standpoint of what would happen in the crystal.

Whatever may be the outcome of such an approach, it is certain that a good deal of confusion has been introduced in the past by the failure to recognize that the mammalian erythrocyte can assume a variety of metastable forms, many of which are reversibly convertible into each other. The conditions

<sup>9</sup> The idea that the smallness of the volume changes is due to there being bound water in the cell may still turn out to be useful, if by bound water we mean water whose properties are modified as a result of its being a component of a paracrystalline, or in the extreme case, a crystalline structure. While almost all the water in the normal discoidal red cell is believed to be free (Macleod and Ponder, 1936), nothing is known as to the corresponding state of the water in the paracrystalline red cell.

TABLE V



1. The usual form of spontaneous crenation. Probably due to internal changes: local orientation of hemoglobin (Teitel-Bernard). Diminution of  $R$ -value in hypotonic solutions.
2. Observed in the cold rat red cell. A continuation of process 1. Strong birefringence.  $R$ -values in hypotonic systems approach zero.
3. Final stage of processes 1 and 2. Probably not reversible.
4. Initial stage of (a) action of most lysins, (b) action of rose bengal and lecithin, and (c) of slide and coverglass sphering. Probably initially due to changes at cell surface. No volume changes.
5. Continuation of process 4. Easily reversed in case (c) by adding antisphering substance or lowering pH, and in case (b) by adding plasma or by washing. Difficult to reverse in case (a).
6. Effect of hypotonic media: volume increases with  $R$ -values depending on the initial condition of cell (normal disk, crenated disk, paracrystalline disk).
7. *Sudden* change to spherical form, which gradually becomes a prolytic sphere.
8. Lysis of prolytic sphere at a critical volume which depends on the kind of cell and the nature of the environment.
9. Formation of prolytic sphere with the continued action of lysins, and eventual hemolysis. These stages probably irreversible.
10. Change due to variation in  $O_2$  tension in the case of the discoidal promeniscocyte, a genetic variant of the red cell. The meniscocyte or sickle cell is probably paracrystalline;  $R$ -values small in hypotonic media.

under which these metastable forms occur are now sufficiently well defined for us to be able to summarize them as shown in Table V.

Corresponding to these variations in form, there may be a number of states of hemoglobin<sup>10</sup> and of the material which constitutes the cell's fixed framework<sup>11</sup> which have still to be defined, and it is possible that in speaking of red cell structure we shall have to distinguish between a variety of structures, perhaps one for each metastable state. The red cell which undergoes these form changes is certainly more structurally complex than the model which is used in setting up the simple expressions which are supposed to describe its osmotic behavior quantitatively. Changes in a cell membrane alone seem inadequate to account for the varied phenomena observed in connection with red cell behavior, the explanation of which appears to require a more detailed knowledge of the molecular architecture in the interior of the cells.

#### SUMMARY

When washed rat red cells are kept in 3 per cent sodium citrate at low temperatures (4–9°C.), their resistance to osmotic hemolysis increases so that after several days they swell very little in hypotonic solutions ( $R = 0.15$  to zero) and do not hemolyze even in distilled water. In this and in other respects they behave as if they were gelated or paracrystalline. The paracrystalline state is reversible, disappearing when the cells are warmed and rapidly reappearing when they are cooled, and the resistance to hypotonic hemolysis is not due to the cells reaching equilibrium with their environment by losing so much K that the concentrations become equal inside and out. The concentration of K remains about 25 times as great inside the cell as outside it in a hypotonic medium of  $T = 0.1$ , and the failure to swell and to hemolyze seems to be due to the activity of K in the interior of the paracrystalline cell approaching zero. The paracrystalline red cells are more resistant to saponin and digitonin hemolysis, and do not undergo the usual shape transformations, probably because they are too rigid. Hemolysis by saponin and similar lysins occurs without sphere formation, and after lysis is complete a granular debris is left behind. The paracrystalline cells show a diffuse birefringence with polarized light; on their being warmed, the birefringence disappears except at foci which are usually situated along the rim of the cell.

The occurrence of the paracrystalline state accounts for the different amounts of swelling of red cells which have been observed in systems of the same degree of hypotonicity, and its relation to other metastable states of the red cell is

<sup>10</sup> Dr. Drabkin (private communication) tells me that gel formation can be observed in human hemoglobin solutions containing about 63 gm. per cent of hemoglobin. The gel is presumably a state intermediate between that of the crystal and that of the hemoglobin solution.

<sup>11</sup> Beams and Hines (1944) have shown that the red cells of the rat are composed of at least three discrete substances which differ in their relative specific gravities, and which can be stratified by ultracentrifugation.

discussed in connection with a tabulation of the metastable states of the mammalian red cell and their relation to one another. Changes in a membrane alone seem inadequate to account for the varied phenomena observed in connection with red cell behavior, the explanation of which appears to require a more detailed knowledge of the molecular architecture of the cell interior.

I wish to thank Dr. Vincent Dole for his many suggestions and helpful criticism.

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# ON THE NATURE OF FORCES OPERATING IN BLOOD CLOTTING

## I. THE PARTICIPATION OF ELECTROSTATIC ATTRACTION

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### 1. INTRODUCTION

Although a large number of investigations have been carried out with the purpose of throwing some light on the mechanism of the so called second phase of blood clotting, the transformation of fibrinogen into fibrin under the influence of thrombin, it must be admitted that our actual knowledge in this field is very slight. In the complex of problems mentioned one can distinguish three main questions: (1) What is the nature of the reaction between thrombin and fibrinogen; is thrombin an enzyme? (2) What kind of forces cause the insolubility of fibrin in contrast to the solubility of its precursor? (3) What details may be described from the point of view of macromolecular morphology? In this series of papers the first two questions will be dealt with. First, some theoretical considerations will be discussed (sections 1 and 2).

Fibrinogen is, under suitable conditions, a soluble protein with thread- or rod-shaped molecules. Fibrin is a gel. The difference between a gel and a sol of linear molecules is that in a gel the interference between the anisometric particles is so strong that the system is able to exert a certain resistance against mechanical deformation. A linear sol can change into a gel by strong elongation of the particles or by a meshy association between the long particles by new bonds. A gel without fixed connections between the particles can take up an unlimited amount of solvent. A gel with connective bonds on the other hand can take up only as much solvent as the structure allows. Under normal conditions the swelling of fibrin is of the limited type.

Recently Signer and von Tavel (8) studied an interesting case of the genesis of a gel with limited swelling: the mutual linkage of molecules of methylcellulose with oxalyl chloride. So the solution of the methylcellulose is, by means of main valency bridges, transformed into a gel with a definite structure. In certain respects one can regard methylcellulose and oxalyl chloride as models of fibrinogen and thrombin respectively.

It must be remarked, however, that the forces which limit the possibility of swelling need not to be of the nature of main valency bridges. The forces operating in the class of phenomena known as "coacervation" (see section 2)

are likewise able to form connections of sufficient strength. A gelatin gel for instance shows limited swelling in a solution of an alcohol of a concentration which causes coacervation of liquid gelatin (unpublished data). This is a point of importance for biology, because the coacervation forces are easily affected by external factors, and therefore may be varied much more easily than main valencies. In protoplasm, the intensity of the binding forces changes continuously in connection with the phenomena of life.

What kinds of forces determine the gelatination of fibrinogen during its transformation into fibrin is entirely unknown. A number of facts give the impression that some process of coacervation plays a rôle, and it was with this hypothesis (which turned out to be right) that this investigation was started.

The method of investigating the nature of binding forces in a case such as ours is to study the effect of added substances upon the intensity (strength, number) of the bonds. The method of investigating blood clotting is to observe the clotting time of a system of certain composition. Is there any possibility of connecting these two things?

The first visible clotting occurs if a certain number of bonds  $n$  are formed;  $n$  is a certain fraction  $\frac{1}{p}$  of the number of bonds  $m$  which are maximally possible under the given conditions. As the formation of the bonds is a time reaction the number  $n$  will be reached after a certain time  $t_n$  after the mixing of fibrinogen and thrombin. If we now in another experiment add some substance which weakens the tendency to bond formation, the number of bonds maximally possible,  $m'$ , is now smaller than  $m$ , and the number of bonds  $n'$  formed in the time  $t_n$  is smaller than  $n$ , therefore no clotting is visible. Clotting only occurs if after a longer time  $n$  is reached, which is of course only possible if  $m'$  is not smaller than  $n$ . For  $m' < n$  no clotting occurs at all; the inhibition is then complete. In the range of  $n < m' < m$  the clotting time is a quantitative (though not linear) standard of the weakening of the binding tendency.

Having a means of measuring the binding tendency we can test the hypothesis of the participation of a coacervation process by the criteria developed in the next section.

## 2. Coacervation

Our knowledge of coacervation is almost exclusively due to the extensive researches of Bungenberg de Jong (2, 3). According to him, a coacervate is a condensed system of colloids between which certain attractive (and repulsive) forces act. In a complex or autocomplex coacervate the attraction is due to the presence of positive and negative charges on the components of the colloidal system. In the group of complex coacervates there are two components, one positive and one negative. In the autocomplex systems there is

only one component, bearing a pattern of positive and negative charges simultaneously. If the strength of the attracting forces is sufficient, the system condensates. The visible effect of this depends on the nature of the substances involved: liquid coacervates (for instance drops), gels, films, fibers, etc.

As is generally the case with colloids, neutral salts have the property of decreasing the effective charges. Cations decrease negative, anions eliminate positive charges. Because in a complex or autocomplex coacervate the components are kept together by electrostatic attraction, their stability is decreased by addition of salts.

In the elimination of the charge of colloid particles, polyvalent, or highly polarisable ions are far more efficient than simple monovalent ions. So a negative sol is flocculated more easily by  $\text{CaCl}_2$  than by  $\text{NaCl}$  (valency rule of Schulze-Hardy). In a complex or autocomplex coacervate the attraction is determined by both kinds of charges, so their stability is decreased by salts according to a double valency rule:

$$3 - 1 > 2 - 1 > 1 - 1, \quad \text{and } 1 - 3 > 1 - 2 > 1 - 1$$

(The pairs of numbers indicate the valencies of cations and anions respectively; for instance,  $\text{LaCl}_3$  is a representant of the 3-1 class.) The finer differences between the members of a salt class, for instance between the alkali halides, are caused by the differences in ionic radius, polarisability, and polarising power, and can be understood or predicted on the basis of the theory of the electrostatic bond (as summarised by van Arkel and de Boer, 1).

We can refine these statements by some quantitative derivations making use of potential curves (Fig. 1). We consider the simplest case of a mixture of positive and negative dissolved particles of a charge  $Z e$  in a medium of the dielectric constant  $\epsilon$ . The attractive force between them is, according to Coulomb:

$$K = \frac{-Z_1 Z_2 e^2}{\epsilon a^2} \quad (1)$$

in which  $a$  is the distance between the particles. The energy which is gained when two particles approach each other up to a distance  $r$  is calculated as:

$$A = \int_{\infty}^r -K da = \int_{\infty}^r -\frac{1}{\epsilon} \frac{Z_1 Z_2 e^2}{a^2} da = -\frac{1}{\epsilon} \frac{Z_1 Z_2 e^2}{r} \quad (2)$$

The energy of the attraction is therefore proportional to  $\frac{1}{r}$  (curve A in Fig. 1).

The repulsion includes effects of different nature. In earlier years Bungenberg de Jong tried to explain it by the repulsion between the hydration

orbits. As no such a hydration exists, it cannot be used for our purpose. At greater distances (such as those prevailing in liquid coacervates) it is mainly the diffusion tendency which acts toward preventing aggregation. We can calculate its effect most easily on the roundabout way *via* osmotic pressure. (Compare Nernst's well known derivation of the diffusion law.) In the case of ideal solutions, osmotic pressure is proportional to concentration, that is,

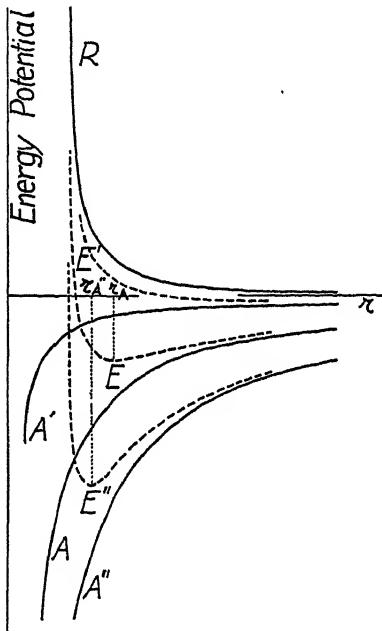


FIG. 1. Theory of complex coacervation Explanation in the text.

inversely proportional to the third power of the mean distance between the particles. In more concentrated solutions,  $\pi$  is proportional to  $\frac{1}{r^n}$ , in which  $n > 3$ . At very small distances between the particles real repulsive forces come into action, in which still higher (and increasing) values, for instance  $n=8$ , are valid (*cf.* van Arkel and de Boer; Pauling). For the energy potential of the repulsion we find:

$$R = \int_{\infty}^r \frac{P}{a^n} da = \frac{nP}{r^{n-1}} \quad (3)$$

in which  $P$  is some constant, which is indifferent for the present purpose, and in which  $n = 3$  for large distances, but increases if the particles approach each other (curve R in Fig. 1).

The total energy effect  $E$  is found by addition of the effects of attraction and repulsion:

$$E = -\frac{1}{\epsilon} \frac{Z_1 Z_2 e^2}{r} + \frac{n P}{r^{n-1}} \quad (4)$$

Its dependence on the distance between the particles is shown by curve E in Fig. 1. At a suitable value of the opposite charges the energy effect shows a definite minimum at a certain particle distance  $r_A$ . In such cases coacervation occurs. If now, for instance by addition of neutral salts, the strength of the attraction is diminished sufficiently (curve A'), then the resulting curve E' does not show a minimum; this means that the particles are distributed evenly in the solution, and the existence of the coacervate is impossible. Increase of the attraction by increased charges or by a lowering of the dielectric constant (for instance by addition of alcohol) on the other hand causes an intensification of the coacervation (curves A'' and E''), demonstrating itself for example in a higher resistance against salts, and in a decrease of the particle distance ( $r_{A''} < r_A$ ).

Apart from complex coacervation due to electrostatic attraction other cases exist, in which the affinity between lipoid molecules is the cause of the attraction. These cases need not be considered now.

If one wishes to decide whether complex or autocomplex coacervation plays a rôle in the formation of fibrin from fibrinogen by thrombin, one has to investigate whether neutral salts inhibit the velocity of clotting of a mixture of fibrinogen and thrombin according to a double valency rule.

### 3. Material and Methods

(a) *Fibrinogen*.—Fibrinogen of high purity (95 to 100 per cent) is prepared from cattle blood by a method, in some respects similar to that of Laki (5, 6). The fibrinogen is precipitated from oxalated plasma with ammonium sulfate (0.2 saturated), the precipitate is washed in ice cold NaCl solution (0.7 per cent), dissolved in NaCl solution of the same concentration, precipitated by adding an equal volume of saturated sodium chloride solution, and redissolved in 0.7 per cent NaCl. It is then purified with Laki's adsorption method, and reprecipitated with  $(\text{NH}_4)_2\text{SO}_4$ . It is redissolved then in 0.7 per cent NaCl, and freed from ammonium sulfate by dialysis. The crystallisation, according to Laki, has been omitted, since cattle fibrinogen, once crystallised, is insoluble.

In most experiments I used solutions containing 20 mg. of fibrinogen per  $\text{cm}^3$ . They are pure white and slightly opalescent.

(b) *Determination of Fibrinogen*.—Upon addition of thrombin to a fibrinogen solution, the whole solution solidifies. Under suitable conditions (see Laki, 5, 6) the gel does not stick to the wall of the tube, and can be squeezed out by means of a spatula. The mass is washed several times, dried, and weighed.

In order to determine the purity of the fibrinogen a determination of the total

protein contents is made in another aliquot of the solution by means of trichloroacetic acid.

(c) *Thrombin*.—Prothrombin is precipitated (together with much fibrinogen) from oxalated cattle plasma by pouring out into eight volumes of cold distilled water and adding acetic acid till a pH of 5.3 is reached. The precipitate is then dissolved in Ca-free Ringer solution, and the prothrombin activated by addition of  $\text{CaCl}_2$  and brain thrombokinase. After separation of the fibrin formed the thrombin is precipitated and dried with acetone. Such preparations have a strength of 0.1 to 2.00 (usually 1.0) unit per mg. (see below). By redissolving and repeating the precipitation with acetone the activity can be raised to 5 to 10 units per mg.

Further purification was obtained by a procedure to be published in a paper on thrombin. So preparations of 800 and more units per mg. have been obtained. It is not certain that this is the pure substance, but possible impurities do not affect the results of this investigation, since it was found to be immaterial whether thrombin of 2 units per mg. or of 1000 units per mg. was used. Likewise fibrinogen of lower purity than 95 to 100 per cent gave identical results.

(d) *Determination of Thrombin*.—Thrombin is determined through its effect upon blood, plasma, or fibrinogen solution. In this work I used a simple determination according to Gerendás (unpublished data):

0.2 cm.<sup>3</sup> oxalated cattle blood is mixed with 0.4 cm.<sup>3</sup> 0.7 per cent NaCl solution in one of the holes of a plate of Jena glass, as used in drop analysis (so called "Tüpfelplatte" of Schott, Jena); 0.2 cm.<sup>3</sup> thrombin (in 0.7 per cent NaCl) is added, and the time is noted after which the solution, when a fine glass loop is drawn from it, shows a fibrous appearance. This point can be determined very accurately; if it is 60 seconds, the thrombin solution is said to contain 1 unit per cm.<sup>3</sup>. A unit is therefore the quantity which clots, at room temperature, 1 cm.<sup>3</sup> of oxalate blood (diluted to 4 cm.<sup>3</sup> with 0.7 per cent NaCl solution) in 1 minute. The method is very useful for the study of commercial styptics. For scientific purposes a test in a simpler system with an invariable standard would be better; this problem is under consideration.

(e) *Investigation of Salt Effects*.—In order to test the influence of added substances (in this case of neutral salt) upon clotting, reaction mixtures are prepared as follows:

0.2 cm.<sup>3</sup> fibrinogen, 20 mg. per cm.<sup>3</sup>, in 0.7 per cent NaCl.

0.2 cm.<sup>3</sup> thrombin, about 4 units per cm.<sup>3</sup>, in 0.7 per cent NaCl.

0.4 cm.<sup>3</sup> salt solution, or distilled water (control experiment).

The time is noted, in which the first clotting is visible. The results of experiments in the presence of salts in various concentrations are compared with the clotting time of a control in which distilled water is added instead of salt solution. In all experiments there is a basic concentration of 0.35 per cent NaCl (0.06 equivalent). This concentration has been chosen in order to keep the concentration of NaCl as low as possible, without making the fibrinogen too labile.

The experiments are carried out in reaction tubes of 10 × 100 mm. The kind of glass influences the clotting time. All experiments have been made therefore with tubes of Jena Geraeteglass 20 of Schott. After use they are washed out, cleaned with chromic-sulfuric acid, washed several times, and treated with water vapour.

All experiments were done at room temperature, 20°C.

*4. The Effect of Neutral Salts upon the Clotting of Fibrinogen with Thrombin*

(a) *NaCl and KCl*.—As will be seen from Fig. 2 both salts have the property of inhibiting the clotting. Relatively high concentrations are needed, and the inhibition does not exceed a prolongation of more than ten times. Higher concentrations of about 2 equivalents precipitate the fibrinogen.

(b) *Potassium Halides*.—In the series KCl-KBr-KI we meet with a strong increase in inhibiting power (Fig. 2). This result is easily understandable: the anions are effective by eliminating positive charges. Iodide is

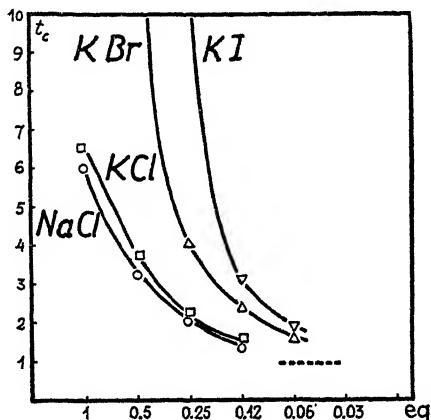


FIG. 2. Inhibitive action of alkali halides on the clotting of fibrinogen with thrombin. Ordinate, clotting time in minutes; abscissa, concentration of the salt in equivalents. The dotted line indicates the clotting time without addition of salts.

more active than bromide, and this more than chloride, because in the halide series the ionic radius increases and the hydration decreases, and because the larger ions are more easily deformable (Table I).

(c) *Bivalent Cations*.—Fig. 3 shows the effects of the halides of Mg, Ca, Sr, and Ba. As will be seen, these 2-1 salts inhibit very much more effectively than the 1-1 electrolytes.

Regarding the differences in activity between these salts, we should expect the series  $Mg^{++} < Ca^{++} < Sr^{++} < Ba^{++}$ , which is the series of increasing ionic radius and polarisability and of decreasing hydration, but the differences will be much smaller than in the potassium halide series, because in the cations the polarisability is much lower than in anions (Table II). Except for an inversion between  $Ca^{++}$  and  $Mg^{++}$ , these suppositions turned out to be right. This inversion is often found in biocolloids, in which the dipole moment (per-

manent + inducible) is of the same order of magnitude as that of water (Bungenberg de Jong and Theunissen, 4).

TABLE I

*Ionic Radius and Polarisability of Some Anions*

	F'	Cl'	Br'	I'
<i>Ionic radius, Å</i>				
Pauling .....	1.36	1.81	1.95	2.16
Goldschmidt .....	1.33	1.81	1.96	2.20
<i>Polarisability, <math>\alpha \cdot 10^{24}</math></i>				
Born and Heisenberg....	0.99	3.05	4.07	6.28
Fajans and Joos .....	0.99	3.58	5.04	7.65

Data cited from van Arkel and de Boer (1).

TABLE II

*Ionic Radius and Polarisability of Some Cations*

Mg <sup>++</sup>	Ca <sup>++</sup>	Sr <sup>++</sup>	Ba <sup>++</sup>
0.65	0.99	1.03	1.35
0.78	1.06	1.27	1.42

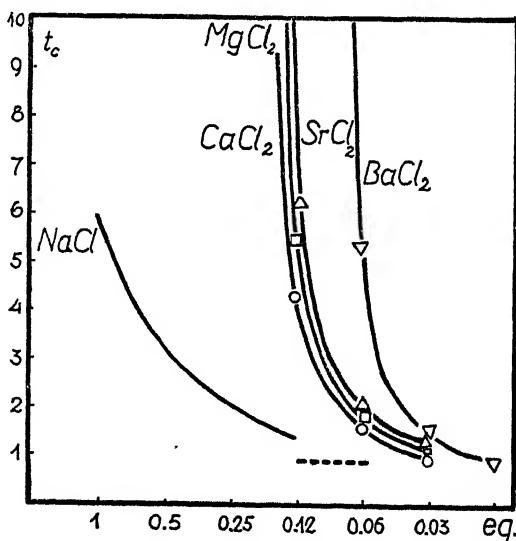


FIG. 3. Inhibition of clotting by bivalent cations. As in Fig. 2.

BaCl<sub>2</sub> shows a somewhat exceptional behaviour. Even in the lowest concentrations used it precipitates part of the fibrinogen. At lower Ba<sup>++</sup> concentrations, however, the remainder of the fibrinogen normally clots, without observable complication on the part of the precipitate. The lowering of the fibrinogen concentration should cause a slight exaggeration of the inhibitory action of the Ba<sup>++</sup>; an eventual binding of a part of the Ba<sup>++</sup> in the precipitate

should cause the opposite. So there is some uncertainty about the exact position of the  $\text{Ba}^+$  curve.

(d) *Polyvalent Anions*.—As in the case of bivalent cations it should be expected that very effective inhibition would be obtained with salts of the 1-n type. As Fig. 4 shows, there exist examples of such behaviour.

However, in this group we meet with complications. As is shown in Fig. 4, sulfates and sulfites (except  $\text{Li}_2\text{SO}_4$ ) are not able to cause inhibition comparable with that of the 2-1 salts. Still more striking is the behaviour of potassium ferrocyanide (1-4) in which case an extremely strong inhibition should

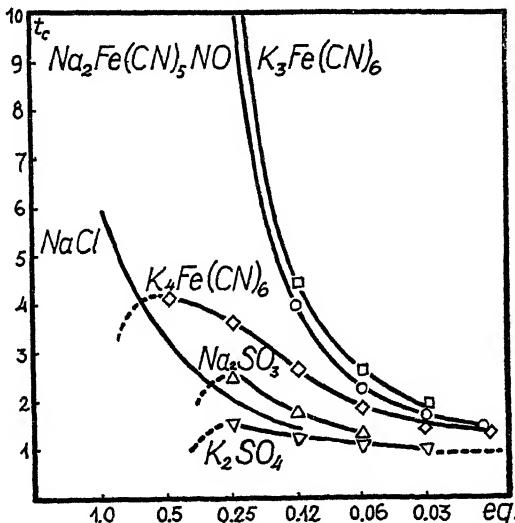


FIG. 4. Inhibition of clotting by polyvalent anions. As in Fig. 2.

be expected. The explanation of these deviations is that all these salts in higher concentrations have the property of precipitating the fibrinogen from its solution, so that they act in the same sense as the thrombin. The cause of this, and the quantitative relations are difficult to understand, because the theory of salting out is still insufficiently developed.

It is, however, possible to eliminate these complications by considering the results of the measurements at very low concentrations. It is true that in these cases no strong effects are obtained, but it is seen that sulfates inhibit at even lower concentrations than the 2-1 salts, and that  $\text{K}_4\text{Fe}(\text{CN})_6$  even inhibits with 50 per cent in a concentration of 0.01 equivalent. The lower concentration limit of inhibitive action is a more important criterion than the maximal inhibition obtainable. So these cases are in perfect agreement with the other experiments.

#### CONCLUSION AND SUMMARY

The results of the study of the inhibiting effect of neutral salts upon the clotting tendency of fibrinogen by thrombin may be summarised as follows:

Salts like NaCl and KCl inhibit only weakly.

Salts of the same cation ( $K^+$ ) with monovalent anions of different ionic radius are the more active the larger the anion ( $Cl^-, Br^-, I^-$ ).

Salts of the same cation with anions of different valency are the more active the higher the charge of the anion ( $1-1 < 1-2 < 1-3 < 1-4$ ).

Salts with the same anion with cations of different valency show stronger inhibition in the case of cations of higher charge ( $K^+, Na^+ < Mg^{++}, Ca^{++}, Sr^{++}, Ba^{++}$ ).

Salts with the same anion and cations of the same charge, but of different radius, are the more active the larger the cation (but with an inversion between  $Mg^{++}$  and  $Ca^{++}$  in the series of the alkali earths, which is not infrequent in bio-colloids).

These results show that the clotting of fibrinogen with thrombin is, at least partly, caused by a coacervation process, due to electrostatic attraction between positive and negative groups. Its nature and localisation will be dealt with in the next paper of this series.

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# ON THE NATURE OF FORCES OPERATING IN BLOOD CLOTTING

## II. THE CLOTTING OF FIBRINOGEN AS A TWO-STEP REACTION

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### 1. INTRODUCTION

In the foregoing paper it has been shown that somewhere in the process of transformation of fibrinogen into fibrin a process of complex or autocomplex coacervation, that is an aggregation due to electrostatic attractions, takes place. Of course this result still allows for a variety of explanations of the mechanism of the clotting process. For instance, it might be supposed that the transition of fibrinogen into fibrin is essentially a denaturation, and that the denatured fibrinogen coagulates because of the liberation of ionogenic groups, which cause an autocomplex coacervation of the particles.

That the clotting of fibrinogen is a denaturation caused by thrombin is admitted rather generally; it is especially propagated by Wöhlsch (10) who considers thrombin to be a specific denaturing enzyme which he therefore calls fibrinogen denaturase. The main argument in favour of this view is a certain similarity in the appearance of fibrin and fibrinogen denatured for instance by heat. It will be clear that this is no real proof; fibrinogen is a rather labile protein, which, in accordance with its anisometric molecular shape, has a strong tendency to form fibrous gels; such gels are always formed when the solubility is decreased sufficiently to cause precipitation; this can be effected by a decrease of the salt contents of the solvent, by salting out, by denaturation, or by thrombin, and the appearance of the precipitate is determined in all these cases mainly by the properties of the fibrinogen. Further there is a synergy between denaturation and the action of thrombin; as both influences have the same effect, *viz.* making the fibrinogen clot, this synergy is quite understandable. None of the arguments of Wöhlsch or other authors give a real proof in favour of the denaturation theory.

In this paper a number of experiments are described which offer another explanation of the mechanism of the action of thrombin, and which make the assumption of a denaturation of fibrinogen by thrombin superfluous and improbable.

### 2. *The Two Steps of the Reaction*

The clotting of pure fibrinogen with purified thrombin is possible only at pH values above the isoelectric point of the fibrinogen (pH 5.3). If, however,

a reaction mixture, containing fibrinogen and thrombin, is kept at for instance pH 5.1 and than neutralised, a normal clot is formed. This shows that at this pH no irreversible damage of fibrinogen or thrombin occurs.

Now it has been discovered by Laki (unpublished data; Laki and Mommaerts, 5) that after neutralisation clotting occurs the sooner (clotting time  $t_c$ ), the longer fibrinogen and thrombin are kept together at pH 5.1 (reaction time,  $t_r$ ). Fig. 1 shows the result of such an experiment; it proves that the

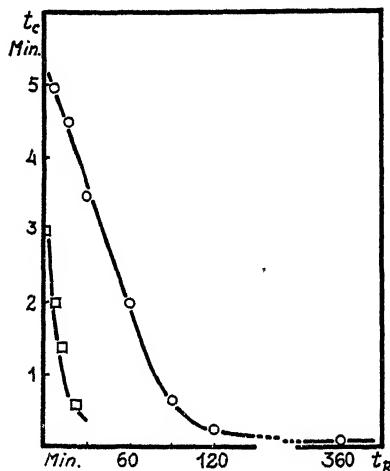


FIG. 1. Explanation in the text.

transformation of fibrinogen into fibrin takes place in two steps. The primary combination between thrombin and fibrinogen, apparently a slow reaction which limits the velocity of clotting, proceeds at pH 5.1, whereas the formation of the fibrin gel takes place only at higher pH values. Of course it is not intended to say that at acid reaction the primary process proceeds with the same speed as in neutral solution.

In this paper, the primary product will be called profibrin. The word profibrin has been used by Apitz (1, 2) to designate the still soluble products appearing in a very slowly clotting solution of fibrinogen with a small quantity of thrombin. As will be discussed elsewhere, these products are a quite heterogeneous mixture, for which no special name may be claimed.

### 3. The Effect of Neutral Salts

The problem arises as to which one of the two steps of the reaction is of coacervate nature. This question may be decided by the study of the effect of neutral salts on the two reactions separately.

The effect of salts on the first reaction may be investigated by making use of the fact that for most salts a certain concentration exists which prevents

clotting completely, whereas a two times lower concentration still allows clotting in a comparatively short time. Of course, the absolute value of that critical concentration depends on the nature of the ions. If now thrombin and fibrinogen are kept together for different times ( $t_r$ ) in the presence of any salt in the critical concentration, and then diluted with an equal volume of distilled water, it is found that again the clotting time ( $t_c$ ) decreases with longer times of reaction (two examples in Fig. 2). This means that the primary reaction proceeded in the presence of the salt, and thus that neutral salts do not inhibit

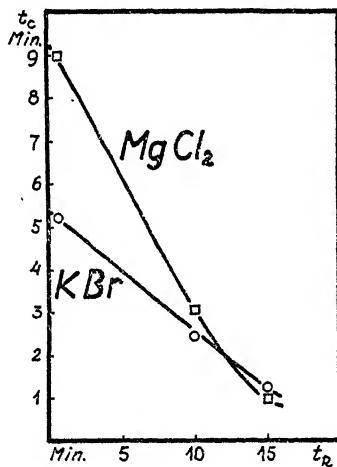


FIG. 2. Explanation in the text.

the primary reaction in a measurable degree. The primary reaction therefore is no coacervation process.

Whether neutral salts inhibit the second reaction may be tested in the following way: thrombin and fibrinogen are allowed to react at pH 5.1, and then neutralised with or without simultaneous addition of salt. An example:

2 cm.<sup>3</sup> fibrinogen, 0.4 cm.<sup>3</sup> thrombin, 10 units per cm.<sup>3</sup>, 2 cm.<sup>3</sup> M/5KH<sub>2</sub>PO<sub>4</sub>; after 20 minutes' neutralisation of 0.4 cm.<sup>3</sup> with 0.2 cm.<sup>3</sup> M/5Na<sub>2</sub>HPO<sub>4</sub>, with and without addition of salts, dissolved in 0.2 cm.<sup>3</sup>.

Some results are shown in Fig. 3. The positions of the curves are not exactly the same as in Figs. 2 and 4 of the foregoing paper. This difference is due to the fact that in the experiments of the kind shown in Fig. 3 the solution contains a fairly high quantity of phosphate ions (~M/15) which likewise inhibit the clotting considerably. Between these and the added ions certain effects of synergy and antagonism occur, which have not yet been studied separately, so that the deviations cannot yet be explained in detail. The general trend of the curves is, however, clear.

We see therefore that the second phase of the reaction, the gelatination of the profibrin, is a coacervation process.

#### 4. Nature of the Primary Reaction

An indication of the possible nature of the primary reaction between thrombin and fibrinogen may be obtained by the study of the inhibition of the clotting process by urea and similar compounds.

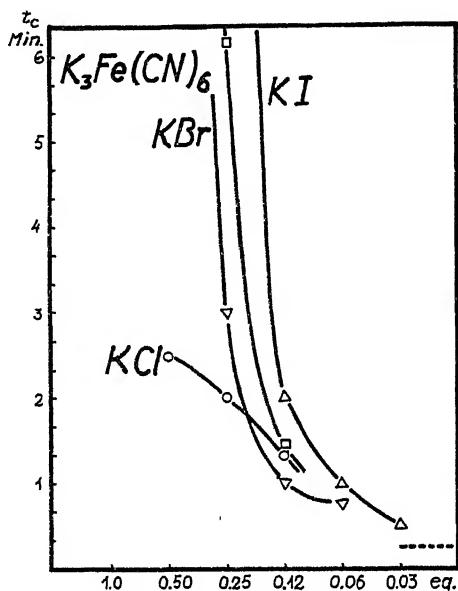


FIG. 3. Inhibition of the second reaction by some salts in different concentrations. The dotted line marks the clotting time without addition of salt.

That urea inhibits the clotting of fibrinogen with thrombin is shown in Fig. 4. Guanidine and other substances have the same effect. The question arises whether the first or the second reaction is affected.

The effect of urea on the primary reaction is investigated in the same way as in the case of salts: a mixture of thrombin and fibrinogen in the presence of urea in a concentration of 10 per cent does not clot, whereas after dilution of the system with an equal volume of distilled water gelatination occurs in a few minutes. The primary reaction can be studied by carrying out this dilution after different periods of time (Fig. 5).

Contrary to the experiments with neutral salts mentioned in section 3 the clotting time is not reduced by long periods of reaction between thrombin

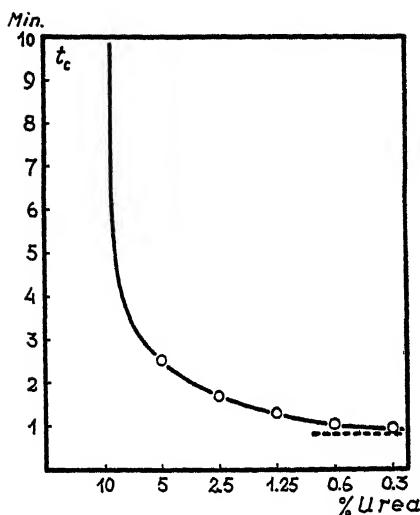


FIG. 4. Inhibition of the clotting of fibrinogen by thrombin by urea. The dotted line indicates the clotting time without addition of urea.

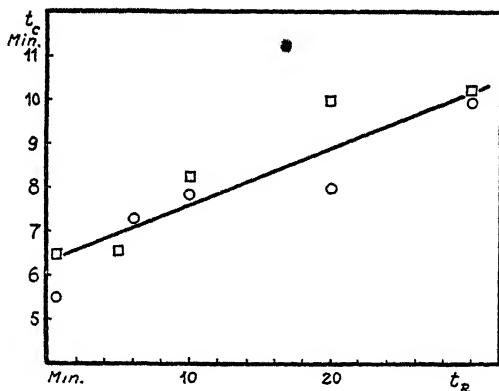


FIG. 5. Inhibition of the primary reaction by 10 per cent urea. For explanation see text.

and fibrinogen. On the contrary, in some experiments the clotting time increased somewhat. That means that in this concentration urea completely inhibited the primary combination of thrombin with fibrinogen.

Does urea affect the second reaction too? In order to get an answer on this question I studied the clotting of profibrin (after neutralisation of a reaction

mixture of thrombin and fibrinogen kept at pH 5.1) in the presence of urea. The following experiment may be cited:

1 cm. <sup>3</sup>	fibrinogen, 20 mg. per cm. <sup>3</sup>
1 cm. <sup>3</sup>	M/5 KH <sub>2</sub> PO <sub>4</sub>
0.3 cm. <sup>3</sup>	thrombin, 3 units per cm. <sup>3</sup>

After different times 0.4 cm.<sup>3</sup> of the solution mixed with 0.2 cm.<sup>3</sup> M/Na<sub>2</sub>HPO<sub>4</sub> and 0.2 cm.<sup>3</sup> distilled water or urea 20 per cent.

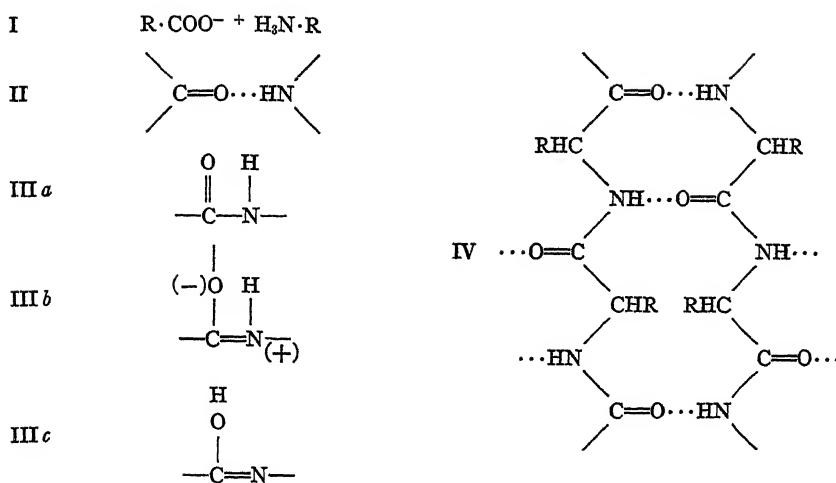
Time of reaction	Clotting time	The same with urea
30 sec.	6 min., 30 sec.	
10 min.	5 " 15 "	
20 "	4 " 30 "	>10 min.
40 "	3 " 10 "	>10 "

It is found therefore that the second phase is inhibited too. In other experiments, however, in which a great deal of thrombin was present, no inhibition was observed. Using a 30 times stronger thrombin solution than in the above experiment, I found that, if the mixture was neutralised after 5 minutes, clotting took place instantaneously, and that urea did not cause an observable inhibition. This could be explained by the assumption that urea inhibits the second reaction in an indirect way, by splitting the profibrin. I found that even a clotting time of 1 to 2 seconds is sufficient to allow for an inhibitive action of urea; an eventual displacement by urea of the thrombin already bound should therefore take place in a very short time. Another possible explanation, however, is that urea directly inhibits the second reaction, and that this inhibition may be neutralised to a certain degree by thrombin in higher concentration. As will be explained in section 6, this assumption is not in disagreement with the fact that the second reaction is caused by electrostatic attraction.

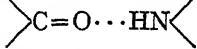
##### 5. *The Nature of Thrombin Action*

In connection with the results dealt with in section 4 some remarks must be made concerning the aggregative forces operating between two parallel peptide chains.

In recent years some investigators (for instance Lotmar and Picken, 6; Mirsky and Pauling, 7) expressed the view that in a protein molecule the peptide chains are kept together (or kept in a folded state) by hydrogen bridges. With that term usually two different kinds of bonds are meant: attraction between a dissociated carbonyl and a dissociated amino group (formula I), and a resonance bond between the >C=O and the >NH- groups of two carbon amide structures (formula II).



In this paper only the second group will be called a hydrogen bond, because the first type may better be considered as an electrostatic bond.

Recently a detailed study of the reactivity of the carbon amide structure has been made by Arndt (3). He found that substances containing this structure (formula IIIa) generally do not have the tendency to complete tautomerisation; that is, of electromery + prototropy (IIIc). Unlike the situation in the case of keto-enol tautomerism, however, there is a possibility of electron displacement without prototropy (IIIb). The formation of IIIb implies the appearance of a dipole and asks therefore for additional energy, so that in isolated molecules this constellation will not occur. In the solid state, however, neighbouring molecules are linked through  bridges, the energy of which suffices to stabilise the configuration IIIb.

Between two peptide chains, or between two parts of a folded peptide chain, formation of hydrogen bonds can take place in the same way (formula IV).



Urea, which disaggregates proteins, likewise possesses the  $-\text{C}=\text{N}-$  structure, and may be supposed to displace two peptide chains from each other by competition. Guanidine and other substances act in very much the same way. An investigation of other substances has been taken up already by the author.

Returning now to the process of blood clotting, the fact that urea inhibits the primary reaction between fibrinogen and thrombin suggests that in the primary reaction thrombin and fibrinogen are linked together with hydrogen bonds, although I admit that at the present state of knowledge evidence is not yet conclusive.

The nature of the second reaction is understandable by the discovery (to be described in a future paper on thrombin) that the isoelectric point of thrombin is situated far in the alkaline range. At pH values at which clotting is possible, the thrombin molecules have a strong positive charge and profibrin has a pattern of positive and negative charges; it aggregates then as an autocomplex coacervate.

The experiments described in this paper form an argument against the view that the clotting process is a denaturation of the fibrinogen. Coagulation of proteins due to denaturation is generally maximal near the isoelectric point because denaturation consists of an unfolding of the molecule, liberating the internal ionogenic groups of the molecule (*cf.* Haurowitz, 4) followed by an autocomplex coacervation of the stretched particles. Coagulation of fibrinogen with thrombin, however, does not take place near the isoelectric point of the fibrinogen, but only at higher pH values. Further, denaturation of fibrinogen is greatly increased, clotting by thrombin, however, always inhibited by NaCl.

Although the process of clotting can be explained without denaturation, and there exist strong arguments against its participation in the clotting process, I shall not yet exclude the possibility that after the formation of fibrin certain changes might occur in the fibrinogen part of the fibrin, which are similar to denaturation. However, up to now there have been no arguments in favour of such a view.

#### *6. The Mechanism of the Clotting Process*

As has been shown in the foregoing paragraphs, the first event in the clotting of fibrinogen is the formation of a compound of fibrinogen and thrombin, called profibrin. This reaction can be studied separately at pH 5.1; it does not cause any physical change of the fibrin solution which might give an indication of a beginning polymerisation, or of a change of form of the fibrinogen molecule.

At pH values at which clotting is possible fibrinogen is negatively, thrombin positively charged and the profibrin has a pattern of positive and negative charges. As in the formation of an autocomplex coacervate, these changes cause an aggregation of the profibrin by electrostatic attraction.

One must keep in mind, however, that these electrostatic forces are not the only attractions operating between two profibrin particles. The numerous

$$\begin{array}{c} \text{O} & \text{H} \\ \parallel & | \\ -\text{C} & -\text{N}- \end{array}$$

groups likewise attract each other; this attraction alone is insufficient to make the fibrinogen insoluble, but it supports the electrostatic effect. By means of salts the electrostatic effect is ruled out, clotting therefore prevented; with urea, however, the attraction of the second kind is decreased, so that likewise the attraction between the profibrin molecules is lowered.

It is therefore not astonishing that at low concentrations of thrombin urea inhibits not only the reaction between thrombin and fibrinogen, but the aggregation of the profibrin too; with much thrombin, however, the electrostatic

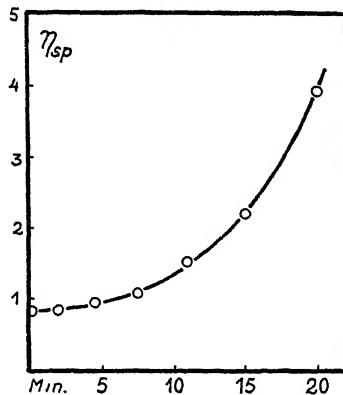


FIG. 6. Increase of the viscosity of a fibrinogen solution, which clots slowly under the action of a small amount of thrombin.  $3 \text{ cm.}^3$  fibrinogen  $\times 0.2 \text{ cm.}^3$  thrombin 2 units per  $\text{cm.}^3$ .

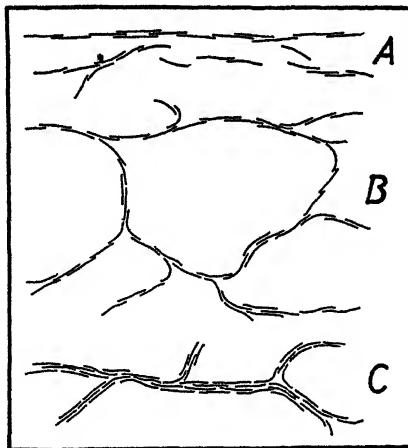


FIG. 7. Schematical representation of the polymerisation of profibrin to fibrin.

attraction dominates to such a degree that now urea hardly affects the second phase of the reaction (section 4). Such a parallel action of electrostatic and hydrogen bonds is not a special property of profibrin. It has been found by the author (9) in the case of aggregated myosin, and in unpublished investigations on the gelatin micelles, and probably the same will be found in other proteins too.

If fibrinogen is allowed to stand with a small quantity of thrombin, so that clotting takes a considerable time, it is found that from the very first moment the viscosity of the solution gradually rises (Fig. 6). Now the viscosity of the solution of an anisometric protein is determined by the proportion  $l/d$  of length and diameter of the molecules (compare the author's analysis of the viscosity of myosin, 8). Thus under the action of thrombin the asymmetry of the anisometric fibrinogen molecules increases. The aggregation of the profibrin thus takes place mainly or exclusively in longitudinal direction (Fig. 7, A).

In further stages of the process the different micelles, while still increasing in length, are more and more connected mutually, till at the end the entire solution solidifies to a gel (Fig. 7, B). But after solidification the reaction proceeds still further, under the formation of crystalline micelles (Fig. 7, C) (the existence of which is proved by Röntgen analysis) till all the thrombin and fibrinogen is exhausted.

#### SUMMARY

It is found that clotting of fibrinogen by thrombin does not occur on the acid side of the isoelectric point of the fibrinogen. At such pH values, however, a primary reaction between thrombin and fibrinogen takes place, leading to the formation of profibrin, a compound of thrombin and fibrinogen.

At pH values at which clotting is possible, fibrinogen is negatively, thrombin positively charged, whereas profibrin has a pattern of positive and negative charges.

The primary reaction, the formation of profibrin by combination of thrombin and fibrinogen, is inhibited by urea but not by neutral salts. The combination of thrombin with fibrinogen most probably takes place by hydrogen bonds.

The second reaction, the polymerisation of profibrin to fibrin, is inhibited by neutral salts in the same way as complex or autocomplex coacervates. It is caused therefore by electrostatic attraction between the positive and the negative charges of the profibrin.

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# PURIFICATION AND PROPERTIES OF DESOXYRIBONUCLEASE ISOLATED FROM BEEF PANCREAS\*

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The isolation of crystalline ribonuclease by Kunitz (1) provided a specific enzymatic reagent which has been widely used in the study of ribonucleic acid. Our interest in obtaining a purified preparation of the corresponding enzyme acting on nucleic acid of the desoxyribose type arose in connection with studies on the chemical nature of the substance inducing transformation of pneumococcal types. Highly purified preparations of the transforming substance isolated from *Pneumococcus Type III* have been shown to consist largely, if not exclusively, of desoxyribonucleic acid, and evidence has been obtained which indicates that the nucleic acid is itself responsible for biological activity (2). Since it seemed desirable to provide confirmatory evidence regarding the nature of the transforming substance by the use of specific enzymatic techniques, a study of the enzyme which attacks native desoxyribonucleic acid was undertaken, especially with a view to obtaining the enzyme in purified form.

The enzyme which breaks down highly polymerized desoxyribonucleic acid has been known for many years under various names. In 1903, Araki (3) reported that crude preparations of trypsin and erepsin brought about liquefaction of gels of  $\alpha$ -thymonucleate prepared according to the method of Neumann (4). Although Araki interpreted the effect as being proteolytic in nature, his experiments represent the first observations on the enzymatic breakdown of this type of nucleic acid. Two years later Sachs (5) demonstrated that liquefaction of the nucleate gel by pancreatic extracts is caused by an enzyme distinct from trypsin, and further presented evidence indicating that trypsin rapidly inactivates the nuclease. De la Blanchardière (6) confirmed the findings of Sachs, and made several unsuccessful attempts to separate the nuclease from the inactivating tryptic enzymes. This writer also devised a more quantitative method of measuring the nuclease activity by following the fall in viscosity of a dilute solution of thymonucleate during digestion with the enzyme.

All of the early workers were somewhat handicapped by the poorly defined character of the substrate used in the enzyme studies. Neumann's method of obtaining  $\alpha$ -thymonucleate from the thymus gland depended on the use of heat and alkali,

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\* The Bureau of Medicine and Surgery of the U. S. Navy does not necessarily undertake to endorse the views or opinions which are expressed in this paper.

which inevitably resulted in a partial breakdown of the material, so that the final preparation consisted of a mixture of hydrolytic products. Experimental demonstration of this partial destruction has been provided by the ultracentrifuge studies of Schmidt, Pickels, and Levene (7) who showed that nucleic acid of the Neumann type sediments with a very diffuse boundary, and that the estimated particle weight varies from 50,000 to above 1,000,000.

The first studies in which a more suitable substrate was used were those of Greenstein and Jenrette (8) who employed sodium thymonucleate prepared by the method of Hammarsten (9). This method avoids the use of drastic reagents, and all procedures are carried out at 0°C. and at neutrality. The preparations so obtained are considerably more homogeneous and highly polymerized than those obtained by Neumann's method. Greenstein and Jenrette were primarily interested in the comparison of the nuclease activity of normal and cancerous tissues, and they investigated a wide variety of organs for the presence of the enzyme, which they now refer to as desoxyribonucleodepolymerase (10). The activity of the enzyme was determined by measuring the progressive fall in viscosity of the nucleate solution when mixed with tissue extracts. It was shown that the enzyme is present in a large number of organs, as well as in the milk and sera of certain mammalian species.

In the several studies mentioned, as well as in others, such as those of Feulgen (11), in which the enzyme was used in investigating the end products of digestion of nucleic acid, relatively crude tissue extracts were employed as the source of the enzyme. In the present paper a method is described for the purification of this enzyme, which for purposes of convenience will hereinafter be referred to as desoxyribonuclease. In addition, certain observations are reported on the chemical properties and kinetics of action of the purified enzyme. The effect of the purified enzyme on the biological activity of the pneumococcal transforming substance will be the subject of a separate communication (12).

## EXPERIMENTAL

### *Measurement of Desoxyribonuclease Activity*

*Source of Enzyme.*—The results of previous investigators indicate that pancreas is by far the richest source of desoxyribonuclease available. Commercial pancreatin was used early in the present study, and was found to provide highly active enzyme preparations. However, attempted fractionation of pancreatin was not particularly successful, and the use of this type of material was discontinued in favor of fresh beef pancreas.

*Substrate.*—Sodium desoxyribonucleate was prepared from calf thymus by the method described by Mirsky (13). In this procedure, minced tissue is thoroughly washed with 0.14 M NaCl and then extracted with 1 M NaCl to obtain the nucleohistone. Purification of the nucleohistone is achieved by repeated precipitation at a concentration of 0.14 M NaCl, and resolution in 1 M NaCl. The histone is then removed by shaking the solution with chloroform and amyl alcohol, the process being repeated until no further chloroform-protein gel is formed. Deproteinization is considerably facilitated by first dissociating the nucleic acid and histone by the addi-

tion of alkali to the 1 M NaCl solution (14). The criterion for dissociation is the lack of formation of a fibrous precipitate when a sample of the solution is diluted with six volumes of water. The deproteinized sodium desoxyribonuclease is precipitated by alcohol, washed with absolute alcohol and ether, and dried *in vacuo*.

A characteristic sample of sodium desoxyribonuclease prepared by this method has a phosphorus content of 8.12 per cent and nitrogen content of 13.5 per cent (nitrogen-phosphorus ratio 1.66). The material dissolves readily in water to give clear, highly viscous solutions which may be stored in the refrigerator for long periods of time without loss of viscosity. Even at higher temperatures, e.g. 37°C., there is no apparent loss of viscosity over a period of several days. The fact that the nucleate solutions show little evidence of spontaneous depolymerization enhances the reliability of a method of measuring enzymatic breakdown based on decrease in viscosity.

*Method of Measuring Enzyme Activity.*—Measurement of the progressive fall in viscosity of nucleate solutions during treatment with the enzyme has yielded constant and reproducible results and has proved a reliable method for determining enzymatic activity. The test is carried out as follows: In all the experiments recorded in this paper Ostwald viscosimeters with a uniform capillary size, giving a flow time for water of from 80 to 100 seconds have been used. 4.8 cc. of a 0.05 to 0.1 per cent solution of sodium desoxyribonuclease in veronal buffer pH 7.5 are introduced into the viscosimeter, which is then placed in a water bath at 30°C. The concentration of substrate is selected so as to give an initial viscosity of not over 3 to 4 times that of water, in order that the rate of flow—and thus the time required for each reading—will not be too greatly prolonged. To 4.8 cc. of substrate, 0.2 cc. of the enzyme dilution is added at zero time, thoroughly mixed with the substrate, and readings of viscosity are made at intervals over a period of about 30 minutes. The enzyme solution under test is diluted so as to give a rate of fall in viscosity that is constant for at least 10 minutes.

The use of a constant external pressure in measuring the viscosity of desoxyribonuclease solutions has been emphasized by Greenstein (8) because of the structural viscosity displayed by these solutions. Under the conditions of the present experiments, in which dilute solutions of desoxyribonuclease are used, the application of an external pressure of 16 cm. H<sub>2</sub>O was found to have little effect on the slope of the viscosity curves or on the reproducibility of the results, and consequently in the interest of simplicity external pressure was not employed in the routine procedure.

*Magnesium Activation.*—In the course of preparative procedures it was observed that the activity of desoxyribonuclease is greatly reduced following dialysis, and that original activity can be completely restored by the addition of magnesium ion to the dialyzed material. Thus, the enzyme is apparently among those requiring a metallic activator. Manganese can replace magnesium and is equally effective at an equivalent molar concentration. Zinc also activates slightly but is less effective than magnesium or manganese. Calcium and iron have no activating effect.

The optimum concentration of magnesium ion is in the order of 0.003 M, and as a routine procedure the enzyme dilutions are made in 0.075 M MgSO<sub>4</sub> so

that the final concentration in the viscosimeter is 0.003 M (*i.e.*, after adding 0.2 cc. of the enzyme dilution to 4.8 cc. of substrate).

*Effect of Varying Enzyme Concentration.*—In initial studies it was found that the effect of varying enzyme concentration on the rate of fall in viscosity of the substrate was irregular and unpredictable. Thus, there appeared to be no regular relationship between the concentration of enzyme and the rate of

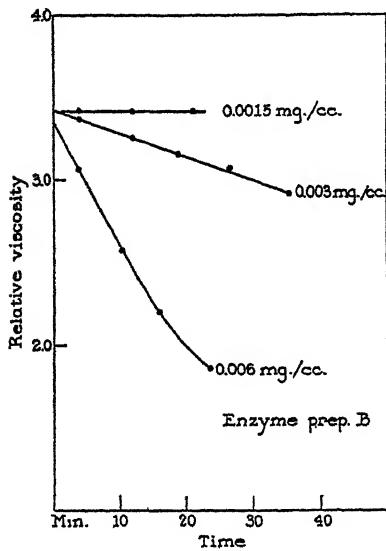


FIG. 1

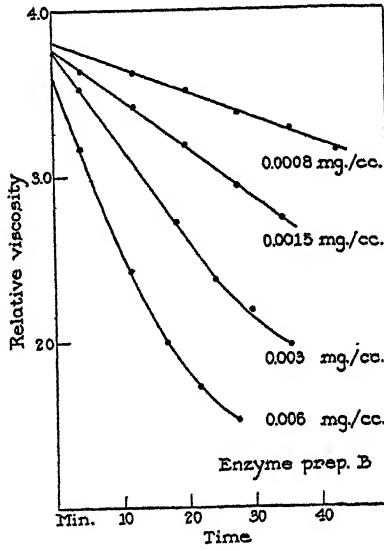


FIG. 2

Figs. 1 and 2. Effect of varying the enzyme concentration and the use of gelatin as a protective agent. Fig. 1 shows the discrepancy in proportionality between the concentration of enzyme and the rate of fall in viscosity in the absence of a protective agent. Fig. 2 illustrates an experiment carried out under the same conditions, except that gelatin was added to the system in a final concentration of 0.01 per cent. In this case a direct relationship is shown between the concentration of enzyme and the rate of fall in viscosity.

reaction, and it was not possible to relate the activity of one enzyme preparation to another on the basis of rate of fall in viscosity. A typical experiment is illustrated in Fig. 1. Twofold decreases in enzyme concentration were made. In decreasing the final concentration from 0.006 mg. per cc. to 0.003 mg. per cc. there resulted a fivefold decrease in the slope of the viscosity curve, and with a further twofold decrease in enzyme concentration activity was no longer detectable. It was thought that this discrepancy in results was probably explicable on the basis of denaturation of the enzyme protein in dilute solu-

tion, and that the addition of a protective colloid to the system might prevent inactivation of the enzyme. Since gelatin has been used successfully as a protective to prevent or retard loss of activity in the case of other enzymes, such as tyrosinase (15) and ascorbic acid oxidase (16), its effect was tested in the desoxyribonuclease system. An experiment in which gelatin was used as protective agent is recorded in Fig. 2. The enzyme preparation and concentrations were the same as those of the preceding experiment (Fig. 1.) The enzyme diluent contained, in addition to the  $MgSO_4$ , gelatin in a concentration

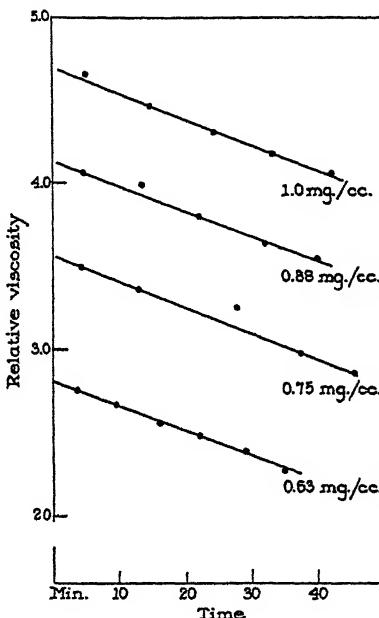


FIG. 3. Effect of varying the concentration of the substrate, sodium desoxyribonucleate.

of 0.25 per cent. The final concentration of gelatin in the viscosimeter was therefore 0.01 per cent, and this amount of gelatin exerted no measurable effect on the viscosity of the substrate. The effect of gelatin in protecting the enzyme was striking. In contrast to measurements carried out without gelatin (Fig. 1), twofold reductions in enzyme concentration resulted in a corresponding twofold decrease in the slope of the viscosity curve (Fig. 2).

Thus in a system containing gelatin the inactivation of the enzyme is sufficiently reduced so that a direct relationship is shown between the concentration of the enzyme and the rate of fall in viscosity, and it is possible to characterize the activity of a given enzyme preparation from the slope of a single curve.

In some later experiments 1 per cent neopeptone has been used in place of gelatin. Identical results are obtained in terms of activity, and indeed very dilute solutions of the enzyme retain activity for longer periods of time in neopeptone than in gelatin. (See below.)

*Effect of Varying the Substrate Concentration.*—The effect of varying the substrate concentration was studied only within a relatively narrow range in which the viscosity of the solution allowed rapid measurement of enzyme activity. An experiment in which the same enzyme preparation was tested against sodium desoxyribonuclease in concentrations of 0.63, 0.75, 0.88, and 1.0 mg. per cc. is illustrated in Fig. 3. The initial relative viscosity varied from below 3 to almost 5. The slopes of the curves at the various concentrations are identical, showing that the rate of fall in viscosity is independent of the substrate concentration within these narrow limits. Thus, under the conditions of the test, the concentration of the enzyme is the limiting factor in determining the rate of fall in viscosity.

In summary, the activity of the enzyme is tested against a 0.05 to 0.1 per cent solution of sodium desoxyribonuclease in  $M/40$  veronal buffer pH 7.5 at  $30^{\circ}\text{C}$ . in the presence of 0.003  $M$  magnesium and 0.01 per cent gelatin. For the purpose of comparing the activity of various preparations, one unit of the enzyme has been defined as that amount which causes a fall of 1.0 in relative viscosity in 20 minutes under the conditions described.

#### *Isolation and Purification of the Enzyme*

The major difficulty encountered in purification of desoxyribonuclease was the marked susceptibility of the enzyme to destruction by the action of proteolytic enzymes in the source material. Thus, pancreatic extracts with extraordinarily high desoxyribonuclease activity were readily obtained by extraction of fresh pancreas with water, but the activity was rapidly lost as the proteolytic enzymes became activated. When fractionation procedures were attempted, solutions of the partially purified material proved to be unstable because of the contaminating tryptic enzymes. Therefore, although pancreas is the richest source of desoxyribonuclease, it has the disadvantage of having at the same time high concentrations of proteolytic enzymes, which in turn digest the active nuclease.

This difficulty has been largely overcome by the use of the acid extract method described by Kunitz and Northrop (17) for the preparation of crystalline chymotrypsin and trypsin from beef pancreas. Fractionation of the acid extract results in more effective separation of the nuclease from the proteolytic enzymes, and a simple procedure for obtaining desoxyribonuclease in a purified form has been devised.

Following the procedure of Kunitz and Northrop, fresh beef pancreas is obtained at the slaughter house and immediately immersed in cold 0.25  $N$   $\text{H}_2\text{SO}_4$  to retard

autolytic processes during transportation. The pancreas is passed through a meat grinder, and the ground tissue suspended in two volumes of cold 0.25 N H<sub>2</sub>SO<sub>4</sub>. Extraction is allowed to proceed in the refrigerator overnight, and the suspension is then filtered through cheesecloth. The residue is reextracted with one volume of 0.25 N H<sub>2</sub>SO<sub>4</sub>, and immediately filtered through cheesecloth. The combined filtrates are brought to 0.2 saturation with ammonium sulfate by the addition of 114 gm. of solid

TABLE I  
*Preparation of Desoxyribonuclease*

Fraction	Amount	Total activity Units	Units per mg. protein*
Beef pancreas Extracted with 0.25 N H <sub>2</sub> SO <sub>4</sub> . Filtered through cheesecloth	10 pounds		
Acid extract Brought to 0.2 saturation with solid (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> . Filtered with aid of filter cel	9 liters	3 × 10 <sup>7</sup> †	
Filtrate at 0.2 saturation (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Brought to 0.4 saturation with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	9 liters	3.5 × 10 <sup>7</sup>	450
Precipitate at 0.4 saturation Refractionated twice between 0.17 and 0.3 saturation (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	20 gm. (semidry)	3 × 10 <sup>7</sup>	5000
Final precipitate at 0.3 saturation	3-4 gm. (semidry)	2 × 10 <sup>7</sup>	7-10,000
Dried enzyme	1-2 gm.	1-2 × 10 <sup>7</sup>	7-10,000

\* Protein estimated colorimetrically with phenol reagent using method of Herriott (18).

† The value for total activity of the acid extract is consistently lower than that of the filtrate at 0.2 saturation with ammonium sulfate. This may possibly be explained on the basis of an inhibitor present in the initial extract which is lost on fractionation.

salt per liter of filtrate. Ten gm. of filter cel and 10 gm. of standard super cel are added for each liter of filtrate, and filtration is carried out with suction on a large Büchner funnel. The precipitate is discarded. The clear, straw-colored 0.2 saturated filtrate is brought to 0.4 saturation by the addition of 121 gm. of solid ammonium sulfate per liter, and the resulting precipitate is allowed to settle for 1 or 2 days in the refrigerator. The slightly turbid supernatant is siphoned off as far as possible, and the precipitate recovered on hard filter paper (Schleicher and Schull No. 275). The precipitate at 0.4 saturation contains practically all of the desoxyribonuclease. Chymotrypsinogen, trypsinogen, and ribonuclease are present in the filtrate, which

can be subjected to further fractionation procedures, as described by Kunitz and Northrop (17) and by Kunitz (1) to obtain these enzymes in crystalline form.

The precipitate at 0.4 saturation is redissolved in a small volume of water (50 cc. to each 10 gm. of moist precipitate) and brought to 0.17 saturation by the addition of 20 cc. of saturated ammonium sulfate solution to each 100 cc. of enzyme solution. A small amount of precipitate forms, which is removed by filtration after stirring in 2 gm. of standard super cel for each 100 cc. of solution. The filtrate is brought to 0.3 saturation with ammonium sulfate, and the precipitate formed is recovered on hard paper. This precipitate is dissolved in water and refractionated between 0.17 and 0.3 saturation with ammonium sulfate. Precipitation of the enzyme at the relatively low concentrations of ammonium sulfate used is dependent upon the pH and concentration of the solution. The pH of the solutions from which the enzyme is precipitated at 0.3 saturation is 4.0-4.5, and if the pH is increased to 7 or above, considerably larger amounts of salt are required to bring down the desoxyribonuclease.

The final 0.3 saturated precipitate is dissolved in a small volume of water (10 to 20 cc.) and dialyzed in the cold against 0.002 N H<sub>2</sub>SO<sub>4</sub>. The dialyzed solution is then dried *in vacuo* from the frozen state. A summary of the method of preparation, giving average values for the amount and activity of the various fractions is presented in Table I.

The yield of dried enzyme from 10 pounds of pancreas is 1 to 2 gm. Numerous attempts have been made to crystallize this purified product, but none has so far been successful.

#### *Properties of the Purified Enzyme*

*General Properties.*—The activity of the dried enzyme is well preserved on storage. The dried material dissolves readily in water to yield clear, colorless solutions with a pH between 4 and 5. The enzyme has its maximum stability in this pH range, so that aqueous solutions are relatively stable. At higher pH values, and especially above pH 7, loss of activity is much more rapid, but this instability may be chiefly the result of the action of small amounts of proteolytic enzymes still present in the purified preparations.

Highly dilute solutions of the enzyme are unstable even in the presence of gelatin, and in order to obtain reproducible results it is necessary to measure activity immediately after preparation of the dilution. Increasing the amount of gelatin has not served to augment significantly the stability of the dilute enzyme solutions, but as mentioned above, the activity of the enzyme is remarkably well preserved in complex mixtures such as neopeptone. Because of its complex nature and variable constitution, peptone cannot be considered ideal for this purpose, but it has proved very useful in those cases where stable dilutions are required.

The results of preliminary studies in the electrophoresis apparatus indicate that the isoelectric point of the enzyme is from pH 5.0 to 5.2. One major

electrophoretic component is present with a small amount of at least one other component.<sup>1</sup>

*Specific Activity on Sodium Desoxyribonucleate.*—Purified preparations display high activity as measured by the fall in viscosity of desoxyribonucleic acid. As indicated in Table I, the dried material contains approximately 10,000 units per mg. of protein. Thus, 0.1 microgram of the enzyme represents one unit. A definite effect can be demonstrated with enzyme concentrations of less than 0.01 microgram per cc.

The measurement of activity of the dried enzyme is illustrated in Fig. 4. An aqueous solution containing 5 mg. of enzyme per cc. was diluted 100-fold

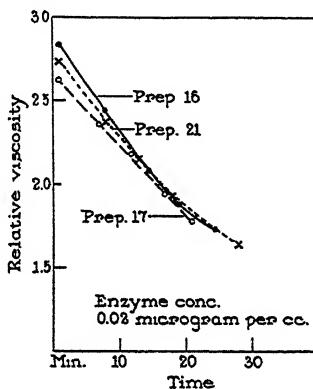


FIG. 4. Activity of purified preparations of desoxyribonuclease.

in water in a volumetric flask. From this dilution a further 100-fold dilution (final 1:10,000) was made in the gelatin-MgSO<sub>4</sub> diluent. 0.2 cc. of this dilution (containing 0.1 microgram of enzyme) was mixed with the substrate as described under measurement of activity. The curves obtained with three separate preparations of the enzyme are recorded in Fig. 4, and serve to demonstrate the uniformity in activity of different lots prepared by the method described.

*Action of the Purified Enzyme on Substrates Other Than Desoxyribonucleic Acid.*—The purified preparations were tested for enzymatic activity on a variety of substrates other than desoxyribonucleic acid in an attempt to obtain information as to the completeness of the separation from other constituents of the pancreatic extracts. Proteolytic activity was measured using gelatin and casein as substrates. The effect on the viscosity of 2.5 per cent

<sup>1</sup> The author is indebted to Lieutenant Commander Vincent P. Dole (MC) USNR, for carrying out the electrophoresis experiments.

gelatin at pH 7.5 was employed as a method of detecting minute amounts of proteolytic enzyme. One to two mg. of purified desoxyribonuclease caused no fall in the viscosity of 5.0 cc. of the gelatin solution, even after attempts to activate the enzyme solution, on the assumption that inactive precursors of the proteolytic enzymes (trypsinogen or chymotrypsinogen) might be present. When 5 mg. of the enzyme were used, a slight effect was noted, corresponding to the effect given by 0.0005 mg. of crystalline trypsin when tested in the same system.

At concentrations of 0.2 to 1.0 mg. per cc. the enzyme also caused definite proteolysis of a 2 per cent solution of casein (pH 7.8) as measured by the increase in tyrosine not precipitable by trichloracetic acid. In general, casein seemed to be much more readily attacked than gelatin. These findings indicate that a small amount of some proteolytic enzyme is present in the purified preparations.

The effect of the enzyme on ribonucleic acid was determined by using the method of Kunitz (1) for measuring ribonuclease activity. A 0.5 per cent solution of yeast nucleic acid was treated for 2 hours (at 37°C. and pH 5.0) with 5 mg. of the enzyme, with no resultant increase in acid-soluble phosphorus. The results indicate that desoxyribonuclease has no action on ribonucleic acid, and this is in conformity with the observations of others on the specificity of ribonuclease, which in turn has no action on desoxyribonucleic acid.

The purified enzyme was also tested for lipase activity using tributyrin as substrate, and for alkaline phosphatase using sodium- $\beta$ -glycerophosphate. In neither instance was any evidence of splitting detected. It is concluded that the purified preparations do not contain significant amounts of lipase, phosphatase, or ribonuclease.

*Optimum pH.*—The optimum pH for the action of the enzyme covers a broad range from 6.8 to 8.2. The viscosimetric method is not suitable for measurement of activity below pH 6, because in the acid range non-enzymatic depolymerization of the substrate occurs.

*Heat Liability.*—In contrast to ribonuclease, which is remarkably heat-stable, desoxyribonuclease is readily inactivated by heat. Experiments carried out with 0.5 per cent solutions of desoxyribonuclease in N/100 HCl, in acetate buffer pH 5.0, and in veronal buffer pH 7.8 gave comparable results. In each case, heating at 55°C. for 15 minutes resulted in a loss of 90 per cent or more of the original activity, and after 1 hour no residual activity was detectable.

*Inhibition of Desoxyribonuclease.*—In the course of studies on the pneumococcal transforming substance, it was observed that crude preparations of desoxyribonuclease from diverse sources were inhibited by sodium fluoride (2). It is now apparent that fluoride inhibition is probably related to the fact that the enzyme is magnesium-activated, since other magnesium-activated

enzymes have been shown to be inhibited by fluoride. A relatively high concentration of fluoride (0.02 to 0.1 M) is required for effective inhibition of desoxyribonuclease under the conditions of the test, and further study of the inhibition is not readily adaptable to the viscosimetric method because the precipitate of magnesium fluoride which is formed interferes with accurate measurement of viscosity. It has not been determined whether phosphate plays an important rôle in fluoride inhibition as it does in the case of the magnesium-activated enolase studied by Warburg and Christian (19).

Citrate forms a soluble complex with magnesium similar to that which it forms with calcium. Because of this fact sodium citrate was tested for its effect on enzyme activity and was found to be a potent inhibitor. In 0.01 M concentration sodium citrate exerts a profound inhibitory effect on the mag-

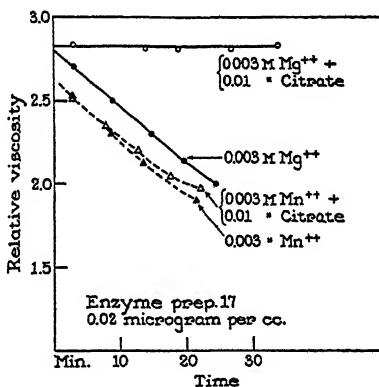


FIG. 5. Effect of citrate on magnesium-activated and manganese-activated desoxyribonuclease.

nesium-activated enzyme even when relatively high concentrations of the enzyme are used. It is of interest, however, that citrate has no such inhibitory effect when manganese is used as the metallic activator. The results of an experiment showing the relationship between the nature of the metallic activator and citrate inhibition are given in Fig. 5. The same enzyme concentration was used in each determination. It is evident that manganese and magnesium ion at 0.003 M concentration activated the enzyme equally. In the presence of 0.01 M sodium citrate, the magnesium-activated enzyme was completely inhibited, while there was no appreciable effect on the manganese-activated enzyme.

Citrate has a similar marked inhibitory action on desoxyribonuclease in crude tissue extracts, and it thus seems likely that magnesium is the naturally occurring activator of the enzyme. In view of this fact, sodium citrate may prove useful as an inhibitor of desoxyribonuclease in biological material. It

has been found, for example, that the enzymatic breakdown of desoxyribonucleic acid which takes place during the autolysis of pneumococci can be completely prevented by citrate without otherwise affecting the course of lysis (20).

*Immunological Data.*—Attempts were made to prepare antisera to desoxyribonuclease by injecting rabbits intravenously with solutions of the enzyme protein. However, after repeated injections by the intravenous route, the sera of the treated animals showed neither precipitins nor inhibitory action on the enzyme. On the contrary, potent precipitating antisera were obtained by the intraperitoneal injection of alum-precipitated enzyme.

A 1 per cent solution of enzyme (preparation 16) was mixed with an equal volume of 10 per cent potassium alum. The pH of the solution was brought to slightly above 7 with N NaOH with the formation of a voluminous precipitate. The suspended precipitate was injected intraperitoneally without washing or centrifugation. Each of two rabbits received a single injection of 10 mg. of enzyme protein by this route. The sera obtained from these animals 1 week later possessed a high titer of specific precipitins. A second intraperitoneal injection of 50 mg. of alum-precipitated enzyme 2 weeks later resulted in a considerable increase in antibody titer.

In Table II are recorded qualitative precipitin tests with the homologous antigen as well as with crystalline preparations of ribonuclease, chymotrypsin, and trypsin which were prepared from a similar acid extract of beef pancreas.<sup>2</sup> The sera were diluted with 1.5 volumes of saline and mixed with an equal volume of the antigen dilutions in saline. Readings were made after incubation at 37°C. for 2 hours and refrigeration overnight.

The data in Table II show that desoxyribonuclease reacts with its antiserum in dilutions beyond 1:1,000,000, indicating a high degree of serological activity for a protein antigen. On the other hand, the crystalline preparations of ribonuclease, chymotrypsin, and trypsin show negligible cross-reactions with the desoxyribonuclease antiserum. Thus, although the desoxyribonuclease is produced by the same organ that elaborates ribonuclease and tryptic enzymes, there is no evidence from the results of precipitin tests that would suggest a serological relationship on the basis of organ or species specificity. These results are in accord with the findings of TenBroeck (21), who showed that trypsin and chymotrypsin from the same species could be distinguished by means of the anaphylactic test. Thus, the incomplete evidence suggests that each of several enzymes making up a part of the external secretion of the same organ have a distinct serological specificity as well as enzyme specificity.

Evidence for the presence of a species-specific antibody in the desoxyribonuclease antisera is afforded by the fact that precipitates are obtained when beef

<sup>2</sup> The samples of crystalline enzymes were obtained through the courtesy of Dr. John H. Northrop and Dr. M. Kunitz of The Rockefeller Institute for Medical Research, Princeton, New Jersey.

serum is used as antigen. However, the amount of precipitate obtained is relatively small. All the cross-reacting precipitins can be removed by adsorption with beef serum, with only a slight decrease in the titer of antibody specifically reactive with the purified enzyme. It is likely that the beef serum reactive antibody is formed as the result of the presence of extraneous protein in the desoxyribonuclease preparation used as immunizing antigen. In this connection it is of interest that antisera prepared against crystalline ribonuclease by Smolens and Sevag (22) showed no evidence of cross-reaction with beef serum. The possibility of cross-reactivity with trypsin and chymotrypsin was not tested by these authors.

TABLE II  
*Precipitin Tests with Antidesoxyribonuclease Rabbit Serum*

Antigen	Rabbit serum	Final dilution of antigen					Saline
		1:10,000	1:30,000	1:100,000	1:300,000	1:1,000,000	
Desoxyribonuclease (preparation 16)	Normal	—	—	0	0	0	—
	Immune	++++	++++	+++	+++	++	—
Ribonuclease (crystalline)	Normal	—	—	0	0	0	—
	Immune	—	—	—	—	—	—
Trypsin (crystalline)	Normal	—	—	0	0	0	—
	Immune	—	—	Trace	Ft. trace	—	—
Chymotrypsin (crystalline)	Normal	—	—	0	0	0	—
	Immune	—	—	V. ft. trace	V. ft. trace	—	—

++++ = marked precipitation with clear supernatant.

— = no precipitation.

0 = indicates that reactions were not carried out at higher dilutions of antigen with serum of the same rabbit, obtained before immunization was begun.

*Effect of Specific Antibody on Enzyme Activity.*—The specific antisera were tested for their effect on the enzymatic activity of desoxyribonuclease. The viscosimetric tests for nuclease activity were carried out as previously described, except that in this instance the final dilution of the enzyme was made in the presence of the antiserum under test, rather than in the gelatin diluent.

A 5 mg. per cc. solution of enzyme (preparation 16) was diluted 1:1000 in the gelatin diluent. A further 1:5 dilution of this solution was made in normal and immune rabbit serum in concentrations of 5 and 20 per cent. The serum dilutions contained 0.075 M MgSO<sub>4</sub>. After 15 minutes at room temperature, 0.2 cc. of the serum-enzyme mixtures was tested for activity in the usual system of buffered desoxyribonuclease with a final volume of 5.0 cc. The final concentrations of serum

were therefore 0.8 per cent and 0.2 per cent, and the final concentration of enzyme was 0.04 micrograms per cc. The tests were carried out in the range of antibody excess, and no visible immune precipitate appeared in either the final enzyme dilution or in the viscosimeter. The results are recorded in Fig. 6.

The presence of 0.8 per cent immune serum resulted in almost complete inhibition of the enzyme as compared with the activity of the same enzyme concentration in the presence of 0.8 per cent normal serum. When the serum concentration was reduced fourfold, the effect was less marked, but about 60 per cent inhibition was obtained. Similar results were obtained on repeated test, and also with serum adsorbed with beef serum, and it would appear that

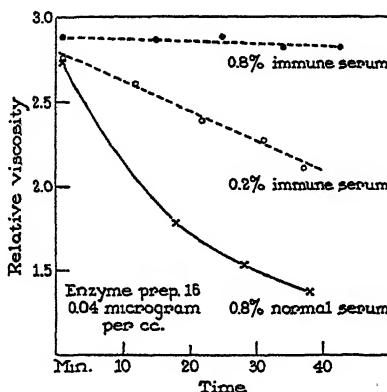


FIG. 6. Effect of specific antibody on the enzymatic activity of desoxyribonuclease.

under the conditions described, desoxyribonuclease falls in that group of enzymes which are inhibited by their specific antibodies.

#### DISCUSSION

The present paper describes the purification and properties of an enzyme isolated from beef pancreas which attacks native desoxyribonucleic acid. This desoxyribonuclease has been obtained in a highly active and relatively purified form, but has not been crystallized. The enzyme is activated by either magnesium or manganese, and its activity is dependent upon the presence of one or the other of these ions in the system.

Previous workers have characterized the action of the enzyme as a depolymerization, since no release of inorganic phosphorus or other readily dialyzable substance occurs, and the end products of the reaction are apparently relatively large in size. Notwithstanding the fact that the enzyme causes only a limited breakdown of the nucleic acid molecule, its primary action is essential for further degradation of this substance by other enzymes. For example, Schmidt,

Pickels, and Levene (7) showed that the high polymer desoxyribonucleic acid prepared by the Hammarsten method was not dephosphorylated by a purified intestinal nucleophosphatase, whereas the latter enzyme could split off phosphorus from the more degraded form of nucleic acid as prepared by Neumann or Levene. After pretreatment of the Hammarsten nucleic acid with pancreatin, until it no longer gave a fibrous precipitate with alcohol, the material was readily dephosphorylated. Thus, enzymatic depolymerization was required before the dephosphorylating enzyme could act.

More recently Wilson (23) has found that strains of hemolytic streptococcus which require certain purines for growth *in vitro* are unable to utilize highly polymerized desoxyribonucleic acid as a source of these purines. However, the same preparation of nucleic acid after hydrolysis by purified desoxyribonuclease supplies the essential growth factor. The streptococcus apparently lacks an enzyme analogous to desoxyribonuclease, but is capable of metabolizing the units which represent the end products of action of this enzyme.

In the light of these and other similar observations, it seems apparent that desoxyribonuclease is a true "nuclease" in that it splits the intact nucleic acid molecule and must act before further enzymatic degradation can occur. For this reason, and in the interest of brevity, the name desoxyribonuclease has been used in this paper in preference to the term desoxyribonucleodepolymerase suggested by Greenstein.

For the purpose of standardizing the conditions, all the experiments described in the present paper were carried out with the highly polymerized form of desoxyribonucleic acid obtained from a single source, calf thymus. However, the enzyme appears to attack indiscriminately desoxyribonucleic acid from a variety of animal and bacterial cells. Similar preparations of desoxyribonucleic acid from beef spleen, shad sperm, and pneumococci are broken down at the same rate and under the same conditions which obtained in the case of calf thymus nucleic acid.

At present there has been no effective procedure developed for removing the traces of proteolytic enzyme which are present as contaminant in the desoxyribonuclease preparations. Fortunately, the activity of the nuclease is sufficiently great so that it may be diluted far beyond the range of detectable proteolytic activity. Thus, while the proteolytic activity of the best preparations can be demonstrated only when 1 mg. per cc. of the enzyme is used, the desoxyribonuclease is active at concentrations below 0.01 microgram per cc. The disproportion between the two types of enzymatic activity is more than adequate to allow practical use of the nuclease under conditions in which proteolytic activity is totally absent. For example, 0.01 mg. per cc. final concentration of the enzyme causes almost instantaneous loss of viscosity of a desoxyribonucleate solution, but at this concentration none of the ordinary protein substrates is attacked.

Detailed investigations of the enzyme in the electrophoresis apparatus or ultracentrifuge have not been carried out, and it is hoped that greater purification of the enzyme can be accomplished by means of crystallization before study of the physical properties or molecular size is attempted. The degree of purification of desoxyribonuclease achieved in the present work, together with the information obtained concerning the properties of the enzyme, is sufficient to provide the specific enzymatic reagent that was sought for use in the study of the transforming substance of Pneumococcus.

#### SUMMARY

1. A method is described for the isolation and purification of desoxyribonuclease from a 0.25 N sulfuric acid extract of beef pancreas. The activity of the enzyme is measured by a viscosimetric method using sodium desoxyribonucleate from calf thymus as substrate.
2. The enzyme is highly active, a measurable effect being obtained at concentrations of less than 0.01 microgram per cc. In highly dilute solution the enzyme is rapidly inactivated, and the use of a protective agent such as gelatin or peptone is necessary.
3. The purified material contains traces of a proteolytic enzyme, but displays no ribonuclease, lipase, or phosphatase activity.
4. The enzyme requires activation by magnesium or manganese ion, and citrate serves as a potent inhibitor of the magnesium-activated enzyme.
5. Its enzymatic activity is inhibited by the specific antibody present in the serum of rabbits immunized with enzyme protein.

*Addendum.*—After the present work was finished and the manuscript completed, a paper came to our attention which describes the purification of the same enzyme under the name thymo-polynucleotidase (Fischer, F. G., Böttger, I., and Lehmann-Echternacht, H., *Z. physiol. Chem.*, 1941, **271**, 246). Although the paper has not been available locally, due to the international situation, a photostatic reproduction has been obtained. The German workers prepared the enzyme from both dried and fresh pancreas. One of the methods described involves ammonium sulfate fractionation of an acid extract of beef pancreas, and in general the procedure is similar to the method presented above, except in the final steps of purification. It is impossible to compare the activity of their preparations with those made in this Laboratory, however, because entirely different methods of measuring activity are employed. The fact that magnesium ion is essential for the action of the enzyme is pointed out, and it is stated that arsenate and arsanilic acid inhibit completely at  $10^{-3}$  M concentration. These workers demonstrated that their preparations had no activity on a variety of substrates other than nucleic acid, including gelatin, ribonucleic acid, nucleotides, nucleosides, and several organic phosphates.

In the light of this article, part of the present paper represents independent confirmation of the work of Fischer *et al.*

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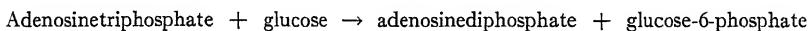
## ISOLATION OF PURE HEXOKINASE FROM YEAST\*

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(Received for publication, November 1, 1945)

Hexokinase, the enzyme which catalyzes the reaction,



has been obtained in pure form from baker's yeast by a method based chiefly on fractional precipitation with alcohol. 1 mg. of the pure protein catalyzes the transfer of 4.2 mg. P per minute at 30° and pH 7.5. The reported crystallization of the protein from dilute alcohol is not readily reproducible. Crystallization from ammonium sulfate (Fig. 1) according to the procedure of Kunitz and McDonald does not change the specific activity of this material.

\* This work was done under National Defense Research Committee Contract No. OEMsr-123 under the auspices of the Office of Scientific Research and Development, which assumes no responsibility for the accuracy of the above statement. The experimental procedure will be published as soon as permission is received from the National Defense Research Committee.



FIG. 1. Crystalline hexokinase from baker's yeast.  $\times 150$ .

# ISOLATION OF CRYSTALLINE HEXOKINASE AND OTHER PROTEINS FROM YEAST\*

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(Received for publication, November 1, 1945)

Hexokinase has been isolated from baker's yeast and crystallized (Fig. 1). Three other proteins of yeast were also crystallized during the course of this work (Figs. 2 to 4). One of these proteins is yellow (Fig. 4) and has a prosthetic group closely related to flavin. No enzymatic activity could be detected in the case of these three proteins.

\* This work was done under Contract No. OEMsr-129 under the auspices of the Office of Scientific Research and Development, which assumes no responsibility for the accuracy of the above statement. The experimental procedure will be published as soon as permission is received from the National Defense Research Committee.

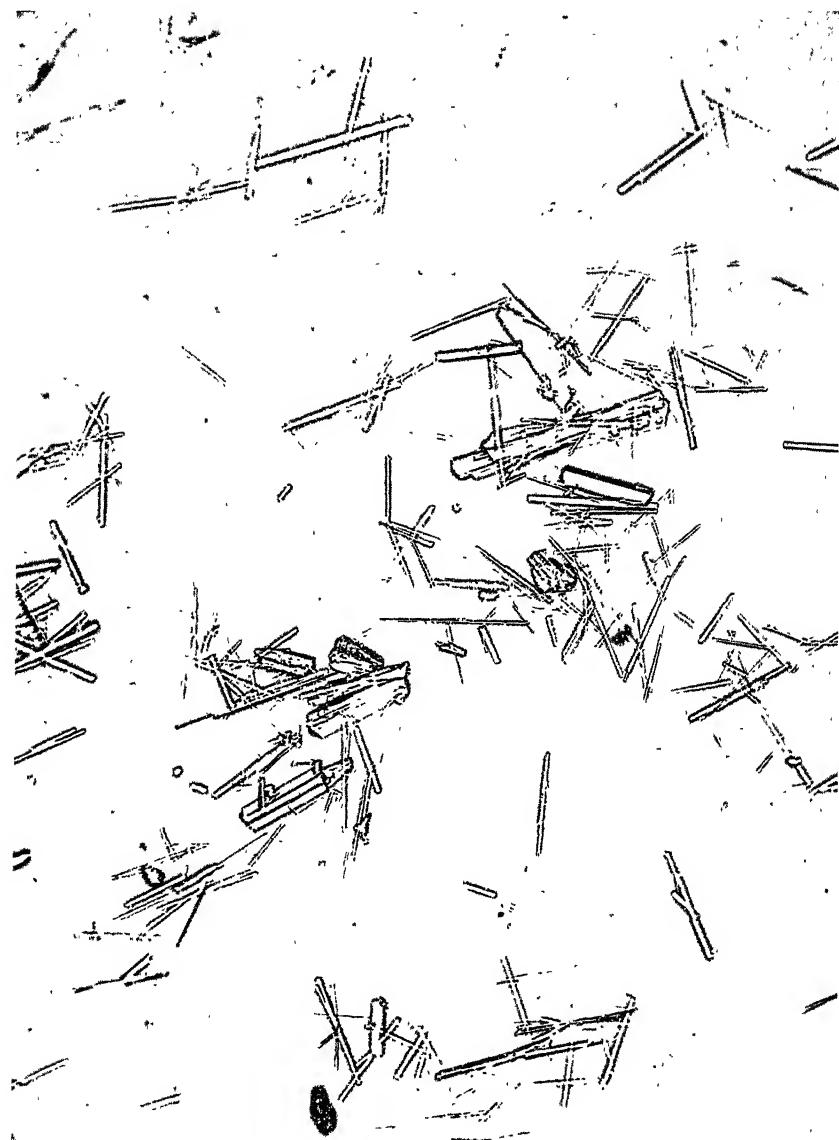


FIG. 1. Crystalline hexokinase from baker's yeast.  $\times 108.2$ .

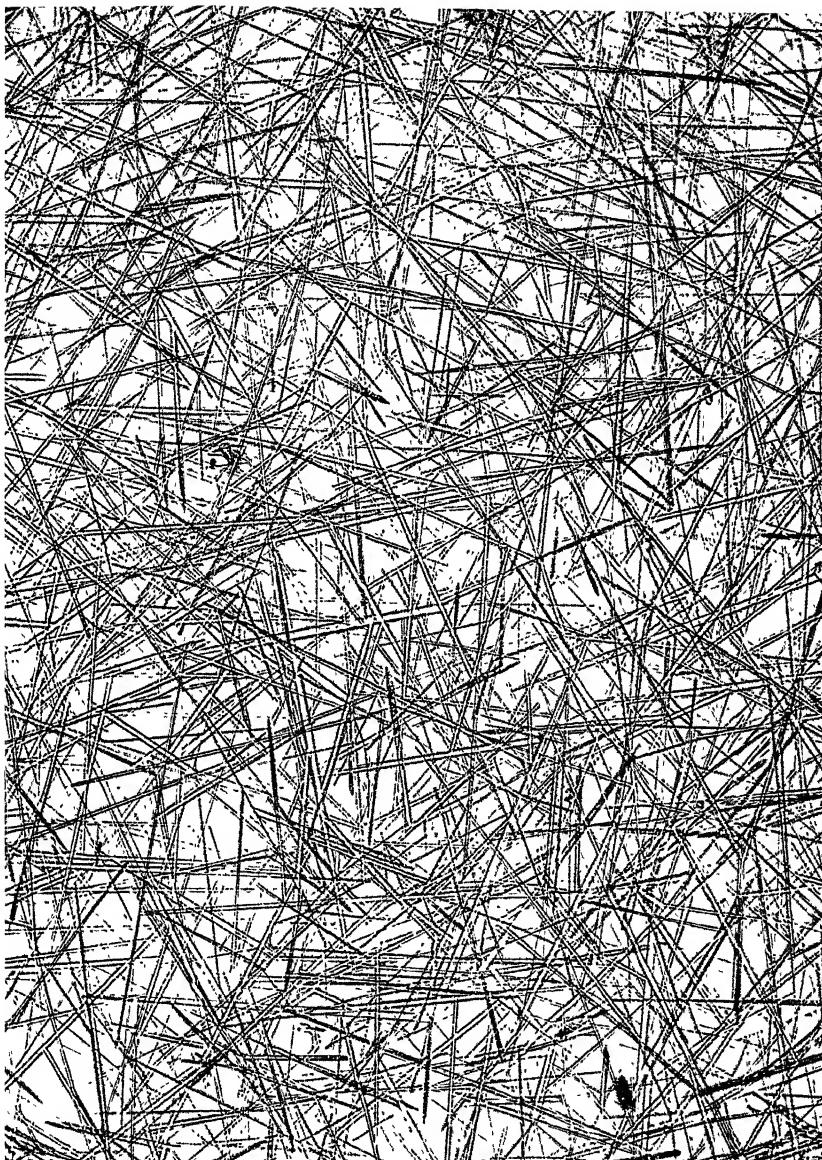


FIG. 2. Yeast protein, No 2.  $\times 129.5$ .

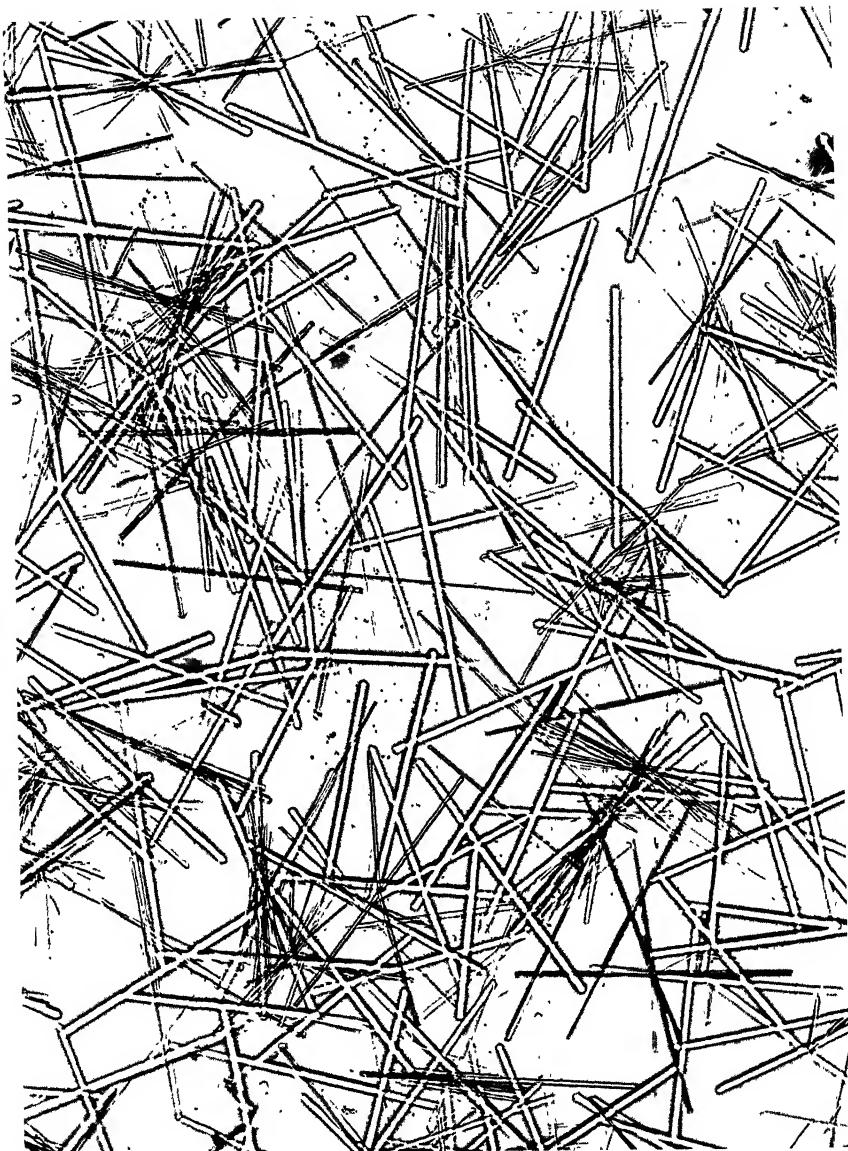


FIG. 3. Yeast protein, No. 3.  $\times 129.5$ .

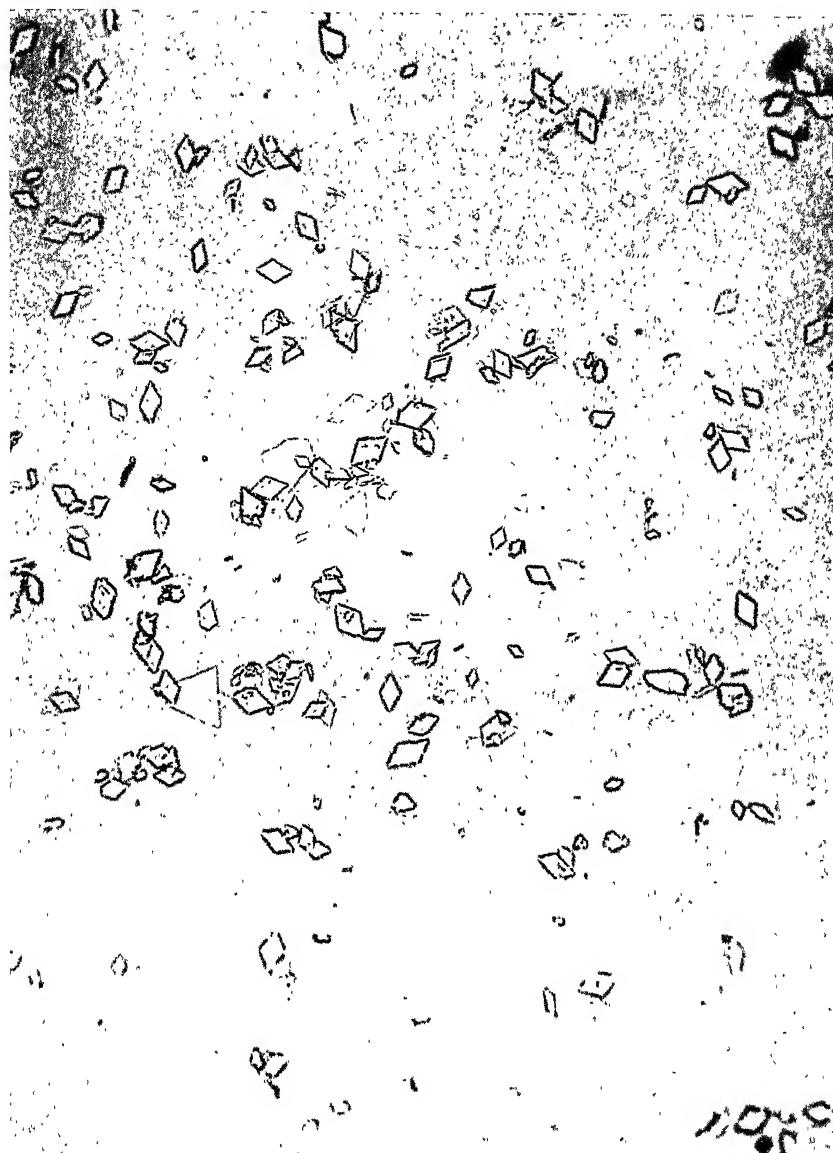


FIG. 4. Yeast yellow protein.  $\times 125$ .



# THE EFFECTIVENESS OF THE SPECTRUM IN CHLOROPHYLL FORMATION

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(Received for publication, October 19, 1945)

## PROBLEM

Light is essential for chlorophyll formation in higher plants, and it is likely that this process involves the building up of a light-sensitive precursor in the dark, and the subsequent formation of chlorophyll upon radiation. That such a precursor exists has been demonstrated many times since Pringsheim first recognized it in 1874. He observed an absorption band at 620 to 640 m $\mu$  in an extract of etiolated leaves, corresponding to a substance which he named etiolin. Monteverde, in 1893, obtained essentially the same result from a spectroscopic examination of extracts of wheat, maize, and sunflower. He called this red-absorbing pigment protochlorophyll, the name by which it is most commonly known today. Lubimenko (1928) believed protochlorophyll to be a breakdown product of the true chlorophyll precursor, chlorophyllogen, because he was unable to identify protochlorophyll in living plants. Subsequently the protochlorophyll band was observed in living, etiolated leaves of *Zea* plants (Scharfnagel, 1931), establishing it as a natural component of etiolated plants. However, it is not possible to observe directly the conversion of protochlorophyll into chlorophyll in the living plant (Noack and Kiessling, 1929) because the appearance of the strong absorption band of chlorophyll at 620 to 640 m $\mu$  would mask the protochlorophyll band in the same place.

Protochlorophyll has not been isolated in pure state, but chemical studies have been conducted on impure extracts of the inner seed coats of the pumpkin seed, which contain a greenish substance with the protochlorophyll absorption bands (Noack and Kiessling, 1929, 1930). This work reveals the presence of a magnesium-rich molecule with porphyrin-like properties. It remained for Fischer, Mittenzwei, and Oestreicher (1939) working on the same experimental material, to identify the molecule as the phyllin of vinylpheoporphyrin  $a_5$  phytol ester, in other words differing from chlorophyll  $a$  only in lacking two hydrogens in the 7, 8 position.

Nevertheless, the main question concerning the pumpkin seed protochlorophyll still remains unanswered: is this substance the true precursor of chlorophyll, or is it a breakdown product of chlorophyll? Both lines of thought can be found in the literature.

The basic fact remains that in etiolated plants there is a substance which

performs the function of precursor to chlorophyll. It would seem highly desirable to obtain an action or effectiveness spectrum of this pigment. The action spectrum is the reciprocal of the amount of incident light necessary to produce a constant physiological effect at various wavelengths, and when properly investigated is closely related to the absorption spectrum of the pigment. In the absence of isolation and synthesis, information about the chemical nature of a pigment can be gleaned from an effectiveness spectrum with the assurance that the substance studied is functioning in the organism. This technique has been used successfully by Hecht (1921, 1922) in the study of photosensitive pigments of *Mya arenaria* and of the human eye, and by Warburg (1927) in identifying the respiratory enzyme in yeast.

#### PREVIOUS WORK

Attempts to investigate the relative effectiveness of different regions of the spectrum in chlorophyll formation have been made, but because the correct way of studying effectiveness spectra was not properly understood, the results obtained cannot be quantitatively related to the absorption spectrum of proto-chlorophyll (Sayre, 1928; Rudolph, 1933; Strott, 1938; and Seybold and Egle, 1938).

In each investigation the same inadequacies can be found. Relatively large regions of the spectrum were compared in effectiveness with the result that the data have only rough quantitative significance. This is borne out by the fact that in no case were quantitative curves given, but only verbal expressions of the relative effectiveness of the regions studied.

Virtually every experimenter determined the amount of chlorophyll formed after different exposure times, with the exception of Sayre who studied the time of exposure for the first appearance of chlorophyll. Filter combinations of known transmissions were used to investigate this function for three different wavelength regions, and the light source was placed at varying distances from the experimental plants to correct for the differences in energy of the lamp arising from the use of filters. By plotting the amount of chlorophyll formed against the duration of exposure for the different wavelengths it becomes possible to evaluate the relative effectiveness of the various colors by reading off how much chlorophyll is formed for a given time of exposure. Critical examination of curves obtained in this manner shows that the relative effectiveness differs depending on what time of exposure one chooses. Clearly in order for this evaluation to be meaningful and quantitative it must be true regardless of a particular choice of experimental conditions.

The method of studying effectiveness spectra employed by the previous workers is valid only if the effect (concentration of chlorophyll) is linearly proportional to energy, which is not true in this case nor in any other known physiological process. A saturation curve is typically found for such functions

(e.g. Smith, 1938). This being the case, the only other approach to the problem is to study the amount of light necessary to produce a constant physiological effect.

As has been pointed out in the papers of Hecht (1921, 1922, 1924, 1940) the above becomes clear when one examines the meaning of the ratio of absorbed light,  $I_{abs}$ , to incident light,  $I_o$ . The variation of this ratio with wavelength is essentially a description of the absorbing power of a colored substance or, in other words, of its absorption spectrum. In order to obtain a relative evaluation of this ratio at different wavelengths, one must hold either the numerator ( $I_{abs}$ ) or the denominator ( $I_o$ ) fixed and vary the other in a known way. The amount of light absorbed bears a relation to the effect produced; if this relation were linear, one could study the ratio of absorbed to incident light *versus* wavelength by determining the amount of chlorophyll formed (which would be directly proportional to  $I_{abs}$ ) for a constant incident energy. Since this linear relation does not obtain, the only other method is to vary the  $I_o$  for a constant physiological effect. This is in conformity with the photochemical law that a constant absorption (constant number of quanta) corresponds to a constant effect.

Two additional objections can be raised concerning previous work on protochlorophyll effectiveness spectra. One has to do with controlling the duration as well as the intensity of the light exposure. In all the previous work mentioned above, time of exposure was considered equivalent to varying the total energy of the incident light; that is, the assumption was made that intensity times time was constant. It is known and indeed discussed by the same authors that once chlorophyll is formed, it is subject to back reactions which take place in the dark. Thus for a 5 hour exposure one will not get five times as much chlorophyll formed as in a 1 hour exposure, but something less than five. There has been more light but also more time for the back reaction to take place. Since this "dark" destruction of chlorophyll cannot be evaluated, the proper procedure is to eliminate it as a variable by keeping the duration of the exposure constant and varying the intensity.

The fourth objection to the previous work has to do with the problem of screening by other pigments present in the plant. Protochlorophyll is small in concentration as compared with the carotenoids present in etiolated plants, so that in the blue end of the spectrum where the carotenoids absorb heavily one would expect distorted results due to the filtering action of the carotenoids. By introducing an amount of energy of blue light equal to that of red an equivalence is achieved that may be more apparent than real because only a small and indeterminate amount of the incident blue light actually reaches the protochlorophyll molecules. Unless the screening can be evaluated, a meaningful spectrum cannot be attained.

In the experiments to be reported here, it has been possible to show that the

carotenoids do not screen the light from the protochlorophyll molecules. Consequently, a quantitative effectiveness spectrum is described, meeting successfully the shortcomings of the earlier work and providing insight into the chemical nature of protochlorophyll.

#### *Apparatus and Methods*

The apparatus is designed to insure uniform and diffuse illumination for each seedling from all sides. In Fig. 1 a diagram of the setup is shown and consists of the following features: The seedlings are arranged around the rim of a beaker 8 cm. in diameter. The beaker is placed on a glass-topped table, and over this is put a

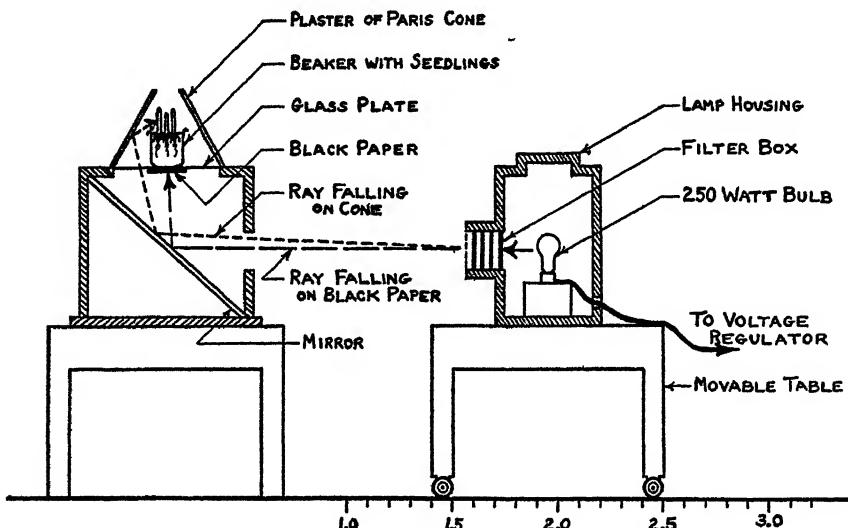


FIG. 1. Diagram of the apparatus used for the light exposure of etiolated *Avena* seedlings.

45° hollow truncated plaster of Paris cone. Below the glass top is a mirror, set at 45°, which reflects upward the light coming from the lamp and filter housing. The glass plate is blackened in the region directly under the beaker so as to exclude any direct light from falling on the plants. This apparatus is similar to that used by Grundfest in his study of the spectral sensibility of the sunfish (Grundfest, 1932).

The housing for the lamp and filters is placed on a movable table so that by varying its distance from the rest of the setup, a variation in the incident energy is achieved. The brightness of the cone surface for different distances of the lamp is obtained with a Macbeth illuminometer. The inverse square law was found to hold, and a calibration curve was constructed relating numbers on the floor and relative energy incident on the plants.

The light source is a 250 watt projection bulb operating through a voltage regulator on 115 volts A.C. This was found to have a color temperature of 2470°K when meas-

ured with an Eastman Kodak color temperature meter, and gave an illumination of 23.66 foot-candles to 0.95 foot-candles depending on its distance from the cone. With the filters in the light beam, the actual illumination used during the investigation is considerably less than this, the filters transmitting only from 1.5 to 60 per cent of the light, the majority of the combinations transmitting 2 or 5 per cent.

Combinations of Corning filters are chosen so that each set transmits only a small spectral region (details in Table V). The transmission properties of each combination

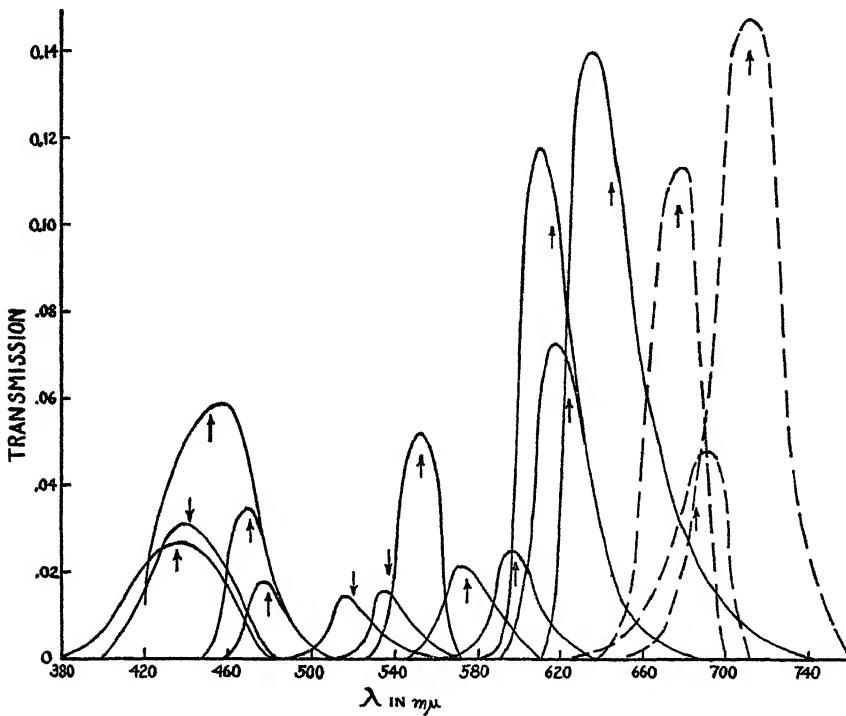


FIG. 2. Relative energy distribution of various Corning filter combinations used with lamp at 2470° K color temperature. Dotted curves are reduced to one-fifth their height (details in Table V).

were measured on the Shlaer photoelectric spectrophotometer (Shlaer, 1938). The visible spectrum was broken up into sixteen overlapping sections each transmitting a range of no more than 40  $m\mu$ , with approximately 75 per cent of the transmitted energy falling within a range of 20  $m\mu$ . By multiplying the filter transmission values for each wavelength by the relative energy content of the lamp of color temperature 2470°K for the same wavelengths (Smithsonian tables), one obtains curves representing the relative energy distribution for each filter combination. These are plotted in Fig. 2. The center of gravity of each curve is determined by plotting these curves on squared paper and counting squares, and the wavelength corresponding to this

point is used as the dominant wavelength. These are indicated in the figure by arrows on the abscissa. In this way, sixteen points covering the spectrum from 436 m $\mu$  to 710 m $\mu$  are found, differing from one another by 6 m $\mu$  to 41 m $\mu$  but averaging a difference of about 20 m $\mu$ . In the region of the peaks of absorption of protochlorophyll an attempt is made to get points close together in order to define the maxima with the greatest possible accuracy.

Suitable filter combinations not being available for the investigation of small regions in the far red end of the spectrum, it is possible to study these areas by using cut-off filters. These are filters which transmit sharply and maximally energy beyond a certain point so that at one wavelength the transmission might be zero, 10 m $\mu$  from that it might transmit 45 per cent of the light, and 75 per cent of the light might be transmitted at the next 10 m $\mu$  increment. By choosing two such cut-off filters which begin to transmit a few millimicra apart, one can evaluate the difference in transmission of the two by plotting the transmission curves on graph paper and counting squares in the area between the two curves. Plotting this difference against wavelength gives a curve of energy distribution similar in shape to that of the other filter combinations. When the effectiveness of these filters in forming chlorophyll is desired, one must determine the effectiveness of each cut-off separately, and subtract the one value from the other. The remainder now become the effectiveness of the difference in relative energy of the two cut-off filters. In Fig. 2 the curves with center of gravity at 676, 686, and 710 m $\mu$  were obtained in this manner.

It is fortunate that these points in the red are attainable because of two conditions found there. The first is that there is a great deal of available energy due to the high transmission of the filters and to the energy distribution of the lamp. The second is that these wavelengths are relatively ineffective in chlorophyll formation. So much energy can be introduced that despite the low effectiveness one can work at a level of chlorophyll formation where the precision in the determination of concentration is best. In some of the regions in the green portion of the spectrum, where the effectiveness is also low, the available energy is limited and this cuts down the precision. The maximum amount of chlorophyll formed under these conditions is so small as to be at the limit of detectability.

The areas under the curves in Fig. 2 are determined by plotting the curves on graph paper and counting squares so that the total relative energy of each combination is known. With this information and the calibration curve of brightness *versus* distance of the lamp from the cone surface, it is possible to evaluate the relative energy of a given filter combination when the lamp is placed at any distance from the experimental plants. In actual practice the calibration curve is plotted as the logarithm of relative energy against the distance in meters on the floor. An arbitrary assignment of the yellow-green filter combination is made to this curve, and the ratio of the area of energy distribution of this particular combination to all the others is calculated. The logarithm of this ratio then becomes the distance either up or down the ordinate (which is in log units) that one must shift the yellow-green calibration curve to be able to read off a value of log relative energy for a particular distance for some other dominant wavelength.

Seedlings of *Avena byzantium* var. *sativa* of three ages, 72, 96, and 120 hours after germination are used as experimental material. These were obtained from the

Bureau of Plant Industry, U. S. Department of Agriculture, and are gratefully acknowledged. Plants of 48 hours are too young to use for pigment studies since the primary leaf is practically undeveloped at this time. The seeds are hulled, soaked for 1 hour in distilled water, and about 40 seeds are placed around the rim of beakers and held in place by a layer of moistened filter paper resting on the bottom of the beaker (Kaiser and Albaum (1939)). Distilled water (350 cc.) is placed in the beakers to insure a high relative humidity. The beakers are kept in the dark in an incubator operating at a temperature of 28°C. ( $\pm 0.1$ ) until the seedlings reach the proper age for experimentation. The beakers are then placed on the glass-topped table of the apparatus in Fig. 1 and exposed to various lighting treatments for 5 hours. During this exposure time the temperature of the dark room was not controlled but did not vary more than 0.5°. Throughout the investigation, the temperature of the room stayed within a 10° range, from 20–30°, and this variation did not affect the chlorophyll formation insofar as could be detected, since similar determinations made at the extremes of this temperature range gave values of chlorophyll concentration within the experimental error.

After exposure to light, the seedlings are placed in boiling water for 1 minute to kill the plants and to minimize the oxidative destruction of carotenoids (Strain, 1938). In all experiments twenty-five seedlings with the longest coleoptiles are selected for extracting. It was found that the longer the coleoptile, the longer the primary leaf, so by selecting seedlings of maximum coleoptile length, one is assured of getting average or above average lengths of primary leaf. For each of the three ages of plants the average primary leaf length for that particular age was determined. For 72 hour plants 15 mm., for 96 hour plants 25 mm., and for 120 hour plants 30 mm. were chosen as the lengths the coleoptile sections were to be cut.

The coleoptiles selected for their long length are cut to this size before extracting, care being taken to obtain a section containing primary leaf throughout. The cuts are made with a razor blade by placing the coleoptile on a white card with parallel lines marked out the proper distance apart. In this manner the maximum length of primary leaf was extracted at each age, which is desirable because of the low concentration of chlorophyll dealt with in these experiments.

When the occasion arose to compare the amount of pigment formed for different ages of plants, a correction was made to account for the differences in the quantity of primary leaf material used at each age. In order to do this, measurements of the total area of primary leaf were made for each aged plant, and the pigment concentrations were expressed for a unit square millimeter area of leaf. The flat surface of the unrolled leaf was measured under a microscope fitted with an optical micrometer device.

The precision of cutting the coleoptiles to the proper length was excellent due to the fact that this operation was conducted under a green Corning safelight. This was chosen because it transmits energy in a region where the human eye is maximally sensitive, but one which is relatively ineffective in chlorophyll formation.

The twenty-five cuttings are ground in a mortar containing Berkshire sand and methyl alcohol. The Berkshire sand was specially cleaned by excess washing in tap water, distilled water, and methyl alcohol to prevent any foreign coloration of the extract. In each extract 8 gm. of sand were used and 20 cc. of Eimer and Amend

c.p. methyl alcohol as the extractive since a comparatively large volume tends to prevent destruction of the pigments (Strain, 1938).

The grinding of the plant sections is timed carefully with a stop-watch. Two minutes of vigorous grinding is done at the beginning of a 1 hour period of extraction at room temperature (20–30° C.) and one-half minute at the end of that time. The primary leaves always appear colorless after this treatment, indicating complete extraction. Whenever light was necessary the green safelight was used.

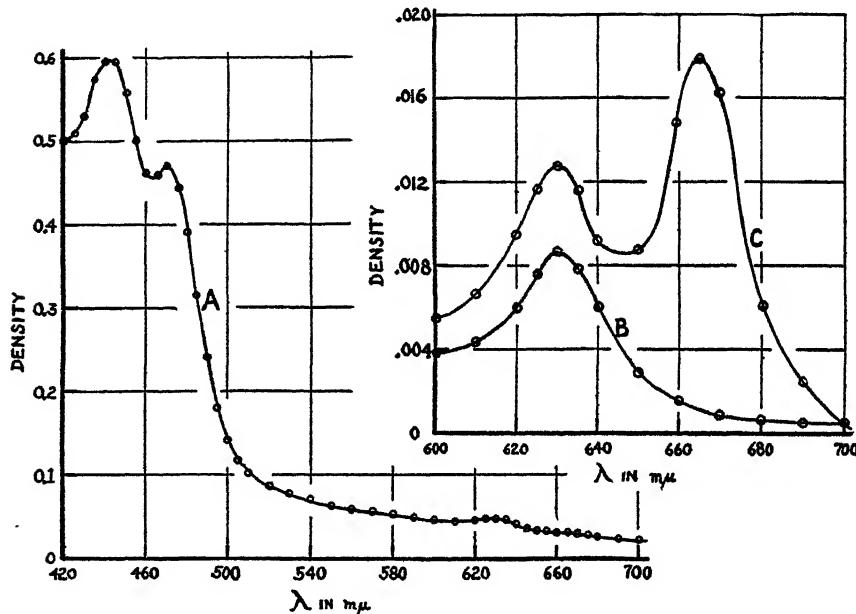


FIG. 3. Absorption spectra of methyl alcohol extracts of total plants: Curve A, in etiolated plants, showing the carotenoid peaks at 440 and 470 mμ; Curve B, red-absorbing portion of curve A on an enlarged scale to show protochlorophyll absorption peak at 630 mμ after correcting for the absorption of the solvent; Curve C, in light-treated plants, showing the chlorophyll peak at 665 mμ.

The extract is filtered with suction, centrifuged for 1 hour at 6°C., and the clear greenish-yellow methyl alcohol solution brought up to constant volume before measuring. This is 15 cc. for 72 hour plant extracts, and 25 cc. for 96 and 120 hour plant extracts, the choice being made in order to work with convenient densities. Here again when it is desired to compare chlorophyll formation for the three aged plants, the chlorophyll values are corrected for this dilution effect.

Immediately afterward the solution is measured spectrophotometrically. It is necessary to time the whole procedure carefully since the pigments are unstable in light and air. Absorption cells 20 mm. in thickness are used with stoppers to prevent evaporation during measurement. The photometric density of the solutions is

measured in the Shlaer photoelectric spectrophotometer, which is especially designed for photosensitive solutions and has a precision of 1/10 of 1 per cent at the region used in these determinations. In this instrument the light passes through two monochromators before being incident on the solution, and the light is of very low intensity so as not to bleach the solution being measured.

Density is equal to  $\log(I_o/I_{tr})$ , where  $I_o$  is the incident light and  $I_{tr}$  is the transmitted light. Since density is proportional to concentration as given by Lambert and Beer's law (Hardy and Perrin, 1932) the density of each pigment was used as a measure of its concentration. In Fig. 3 a typical absorption curve is shown for chlorophyll, protochlorophyll, and carotenoid in extracts of total pigment, showing the density of solution at each wavelength.

In order to evaluate the density of each pigment it is necessary to subtract the absorption of the solvent from that of the solution. Having done this the density of the pigment at its characteristic peak is taken as a measure of its concentration. Thus for chlorophyll, the density at 665 m $\mu$  is chosen; for protochlorophyll (extract of etiolated plants) the density at 630 m $\mu$  is taken; and for carotenoids (extract of etiolated plants) the density at 470 m $\mu$  is chosen. The characteristic 440 m $\mu$  peak was avoided because the precision of the spectrophotometer is not as good here as at 470 m $\mu$ . As the investigation proceeded it was found unnecessary to obtain a whole curve of each extract, measuring the density values at 470, 630, and 665 m $\mu$  being adequate, as well as that at 700 m $\mu$ , the density of the solvent alone.

### *The Screening of Carotenoids*

Before an effectiveness spectrum of protochlorophyll can be studied it is necessary to determine whether the carotenoids act as screens in the blue region of the spectrum where they absorb heavily. To test this, the following experimental plan was devised: first, to evaluate the increase in carotenoid concentration with age; and second, to test the relative effectiveness of blue light and of red light under identical experimental conditions for the three ages of plants. If the concentration of carotenoid increased with age and acted as a screen, one would expect the effectiveness of blue light to drop with increasing age but not that of red. In other words, if the ratio of the effectiveness of blue to red would be found to fall with age, the carotenoids would be acting as screens; if the ratio remained constant, there would be no screening effect.

Measurements on etiolated *Avena* seedlings of three ages (72, 96, and 120 hours after germination) were made to determine the increase in pigment concentration with age. The results are given in Table I. Column 2 gives a sample of the data obtained under the designated experimental conditions, showing the kind of variability between determinations. The data are in density of the primary leaf and equal  $\log(I_o/I_{tr})$ . In order to make the density values of the pigments at the three ages comparable three corrections were made: one to account for the difference in area of primary leaf used in the extracts; a second to correct for the fact that the extracts were diluted to a

different extent before measuring; and a third to account for the fact that the solutions were viewed through 20 mm. absorption cells. Column 3 thus represents values of pigment density of a primary leaf.

It can be seen that the concentration of carotenoid pigment increases significantly with age, but that the increase in protochlorophyll concentration is small and probably not significant. (That carotenoid pigment increases in the leaf has been reported by Rudolph, 1933.) In the 48 hour period from 72 to 120 hours the density of carotenoid has practically doubled.

Having established that the carotenoid concentration increases significantly with age, the question now becomes: does this increased concentration cause

TABLE I

*The Change in Pigment Concentration in Etiolated Seedlings of 72, 96, and 120 Hours after Germination, Expressed As Pigment Density of Primary Leaf*

Carotenoid pigment			Protochlorophyll pigment		
Age	Density of pigment	Average photometric density of primary leaf	Age	Density of pigment	Average photometric density of primary leaf
hrs.			hrs.		
72	0.1288 0.1244 0.1972 0.1544 0.1816 <u>0.1573</u>		72	0.0078 0.0110 0.0078 0.0082 <u>0.0087</u>	
96		0.2160 0.2760	96		0.0119 0.0114
120		0.4200	120		0.0154

a decreased ratio of effectiveness of blue light to red light in chlorophyll formation? Determinations were next made of the amount of chlorophyll formed by plants when subjected to various amounts of light energy for a constant duration. For the three ages of plants, at least four determinations were made of the concentration of chlorophyll formed after 5 hours' exposure to each of four light energies. This procedure was carried out in a red light with dominant wavelength at 645 m $\mu$  as well as in a blue light with dominant wavelength at 442 m $\mu$ . The results of the experiment in red light are given in Table II and plotted in Fig. 4, while those for blue light are found in Table III and plotted in Fig. 5. In each table sample data are included to show the kind of variability obtained.

In the tables chlorophyll concentration is expressed as the average chlorophyll photometric density of the primary leaf. This value is plotted against the logarithm of relative energy in the figures.

TABLE II

*Chlorophyll Formation for Different Relative Energies after 5 Hours' Exposure to Red Light  
(Dominant Wavelength 645 m $\mu$ ) for 72, 96, and 120 Hour Seedlings*

Age hrs.	Chlorophyll pigment formed in red light		
	Log relative energy	Pigment density	Average density of the primary leaf
72	1.37	0.0242 0.0305 0.0330 0.0274 0.0288	0.0396
	1.05		0.0192
	0.78		0.0121
	0.54		0.0038
96	1.37		0.0456
	1.05		0.0256
	0.78		0.0144
	0.54		0.0080
120	1.37		0.0460
	1.05		0.0318
	0.78		0.0190
	0.54		0.0098

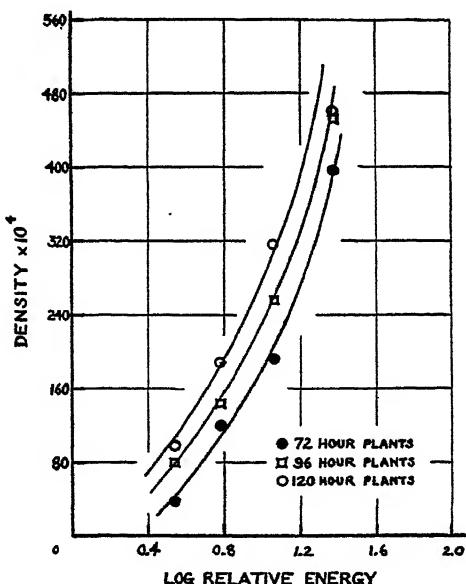


FIG. 4. The chlorophyll density of a primary leaf of three ages of *Avena* seedlings formed after 5 hours' exposure to various energies of red light (dominant wavelength 645 m $\mu$ ).

In order to evaluate the ratio of effectiveness of blue light to red for each age, values for density of chlorophyll at particular values of log relative energy are read off the corresponding curves in Figs. 4 and 5 and the ratios calculated. These values are listed in Table IV. It will be noted that the ratios turn out to be nearly unity; *i.e.*, that blue light and red light are equally effective in forming chlorophyll. This result was obtained purely by chance. A choice of another red or blue would have given different results as will be

TABLE III

*Chlorophyll Formation for Different Relative Energies after 5 Hours' Exposure to Blue Light  
(Dominant Wavelength 442 m $\mu$ ) for 72, 96, and 120 Hour Seedlings*

Chlorophyll pigment formed in blue light			
Age	Log relative energy	Pigment density	Average density of the primary leaf
hrs.			
72	1.37	0.0218 0.0274 0.0366 0.0326 0.0278 <hr/> 0.0292	0.0401
	1.10		0.0222
	0.87		0.0135
	0.40		0.0036
96	1.37		0.0512
	1.10		0.0293
	0.87		0.0184
	0.40		0.0072
120	1.37		0.0494
	1.10		0.0362
	0.87		0.0205
	0.40		0.0062

seen when the effectiveness spectrum is discussed. *The important thing to observe is that the ratio of effectiveness does not change significantly with age.* In spite of a doubling in carotenoid concentration from 72 to 120 hours, the ability of blue light to form chlorophyll is as unimpaired as that of red light for that period. This demonstrates unequivocally that the carotenoids do not act as filters under this particular set of experimental conditions. From a morphological point of view this leads to the unexpected inference that the carotenoids are located behind the protochlorophyll molecules in the plastids.

In Table IV a calculation is made of what would be the expected ratio at

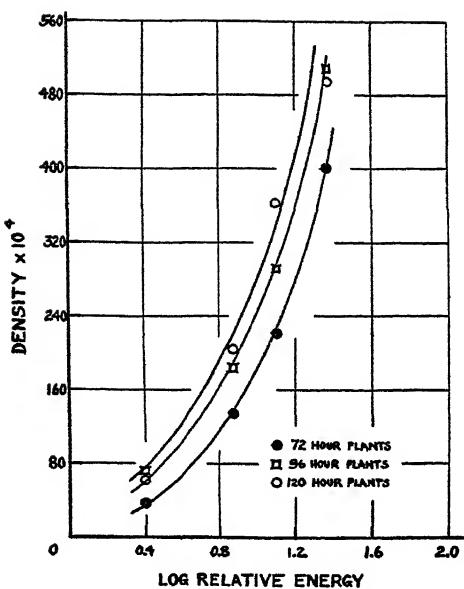


FIG. 5. The chlorophyll density of a primary leaf of three ages of *Avena* seedlings formed after 5 hours' exposure to various relative energies of blue light (dominant wavelength 442 m $\mu$ ).

TABLE IV

*The Ratio of Effectiveness of Blue to Red Light in Forming Chlorophyll at 72, 96, and 120 Hours after Germination*

Age	Log relative energy	Ratio of effectiveness of blue to red (experimental)	Ratio of effectiveness of blue to red (expected if carotenoid acted as screens)
hrs.			
72	0.6	1.250	
	0.8	1.050	
	1.0	0.967	
	1.2	1.010	
	<u>1.4</u>	<u>0.995</u>	
Average.....		1.05	
96	0.6	1.349	1.13
	0.8	1.130	1.08
	1.0	1.080	1.01
	1.2	1.070	1.02
	<u>1.4</u>	<u>1.050</u>	<u>1.02</u>
Average.....		1.14	1.05
120	0.6	1.01	0.82
	0.8	1.01	0.85
	1.0	1.01	0.86
	1.2	1.02	0.88
	<u>1.4</u>	<u>1.03</u>	<u>0.94</u>
Average.....		1.02	0.87

the different ages if the carotenoids had acted as filters. This calculation is accomplished in the following way: The assumption is made that there is a random distribution of carotenoid and protochlorophyll molecules in the plastids. One-half the increment in carotenoid density from 72 hours to 96, and from 72 to 120 hours is thus considered the density of a "filter" placed in the light path, because if the carotenoid molecules were layered in front of the protochlorophyll molecules, all the density increment would filter. If layered behind, none of the increment would filter. A random distribution represents a condition in between these two extremes, or one-half the increment. Consequently, the 96 hour curve in Fig. 5 is shifted toward the right 0.03 log unit, and the 120 hour curve shifted 0.102 log unit. As before, the values of density for particular log relative energies are now read off these shifted curves, and the ratio of these values to the corresponding ones for red light are calculated. The ratio of blue to red falls with age significantly as can be seen in Table IV, column 4. This calculation shows that the experimental precision here is adequate to detect the carotenoid screening effect, had it been present.

#### *The Effectiveness Spectrum*

A proper effectiveness spectrum must record the reciprocal of the relative energy required in different parts of the spectrum to produce the same physiological effect. This energy value can be calculated rather than directly determined providing certain facts are known. First, the relation between chlorophyll formation and relative energy must be known for at least two different wavelengths, because if the curves for any two wavelengths are well defined and are parallel, it is safe to assume that all wavelengths would give parallel curves. And second, the amount of chlorophyll formed for any particular relative energy must be determined for each wavelength region one wishes to study.

By placing both of these data on the same plot, that is the curves of  $\lambda_1$  and  $\lambda_2$  and single determinations of chlorophyll formation for  $\lambda_3$ ,  $\lambda_4$ , etc. one can construct an effectiveness spectrum. By shifting the curve of  $\lambda_1$  along the abscissa (log relative energy) until it superimposes on a single experimental point of  $\lambda_3$ , one can read from the  $\lambda_1$  curve that value of log relative energy for  $\lambda_3$  corresponding to a desired amount of chlorophyll (the ordinate). This saves the work of determining complete curves of chlorophyll formation against log relative energy for each wavelength.

In the experiments reported here only 96 hour plants were used. Consequently, the 96 hour curves of Figs. 4 and 5 were chosen for calculating the effectiveness spectrum. In Table V are found the determinations of chlorophyll concentration for a convenient log relative energy for sixteen filter combinations. Using these data and the curves in Figs. 4 and 5 in the manner described above, the relative energy necessary to form a chlorophyll density

of 0.0280 was found. In Table VI these reconstructed values and their reciprocals are given along with the dominant wavelengths of each filter combination. These are plotted in Fig. 6. There are three definite peaks of "effectiveness," one in the blue, one in the yellow, and one in the red. A suggestion of a peak is present at 545 m $\mu$  and it is drawn as such, because reports on the absorption bands of protochlorophyll in the living leaf viewed spectroscopically (Scharfnagel, 1931) indicate that such a peak is present. There is little other justification for drawing it this way, however, since the

TABLE V  
*Chlorophyll Formation for Various Corning Filter Combinations*

Dominant wave-length m $\mu$	Corning filter combinations	Log relative energy	Chlorophyll density
436	306 + 511	1.43	0.0356
442	038 + 511	1.37	0.0428
452	038 + 554 2 std.*	1.23	0.0265
471	368 1/2 std. + 554 2 std.	1.29	0.0113
480	368 + 554 2 std.	0.96	0.0043
520	503 1/2 std. + 352 + 430	0.90	0.0028
537	348 + 503	0.87	0.0042
552	512 + 350 + 430	1.37	0.0164
575	348 + 430	1.14	0.0159
599	246 1/2 std. + 978 2 std.	1.12	0.0103
615	246 + 978	1.31	0.0280
624	244 + 978	1.11	0.0212
645	243 + 978 1/2 std.	1.37	0.0385
676	$\Delta(244 + 555) - (241 + 241 + 241)$	1.22	0.0222
686	$\Delta(241 + 555 1/2 std.) - (244 + 555)$	1.61	0.0153
710	$\Delta(244 + 585) - (244 + 555)$	2.20	0.0040

\* Standard thickness.

precision of the points in this spectral region is extremely poor. This is due to the small amount of energy available here.

The curve in Fig. 6 may be considered a first approximation effectiveness curve because two refinements can be made. The first limitation arises because of the use of filters and the approximation introduced by using a dominant wavelength for each filter combination. It will be recalled that each combination transmits over a range of 40 m $\mu$ , although most of the light is contributed by a 20 m $\mu$  portion in the center. When the reciprocal of the relative energy is plotted against wavelength in the effectiveness curve, the points on the wavelength scale are placed at the dominant wavelength of the energy distribution curves in Fig. 2. This gives equal weight to all the energy under each curve as far as effectiveness in chlorophyll formation is concerned. When an effectiveness curve obtained with filters contains steep peaks as the

TABLE VI

*Reconstructed Values of Log Relative Energy for a Constant Amount of Chlorophyll Formed  
for Various Corning Filter Combinations*

Dominant wavelength <i>m<sub>μ</sub></i>	Log relative energy	Relative energy	Reciprocal of relative energy
<i>m<sub>μ</sub></i>			
436	1.30	19.95	0.0301
442	1.19	15.49	0.0646
452	1.26	18.20	0.0550
471	1.76	57.60	0.0174
480	1.78	60.30	0.0166
520	1.82	66.10	0.0151
537	1.69	49.00	0.0204
552	1.66	45.70	0.0219
575	1.44	27.60	0.0362
599	1.62	41.70	0.0240
615	1.31	20.40	0.0490
624	1.27	18.60	0.0538
645	1.19	15.49	0.0646
676	1.36	22.90	0.0437
686	1.93	85.10	0.0118
710	3.05	1122.00	0.00089

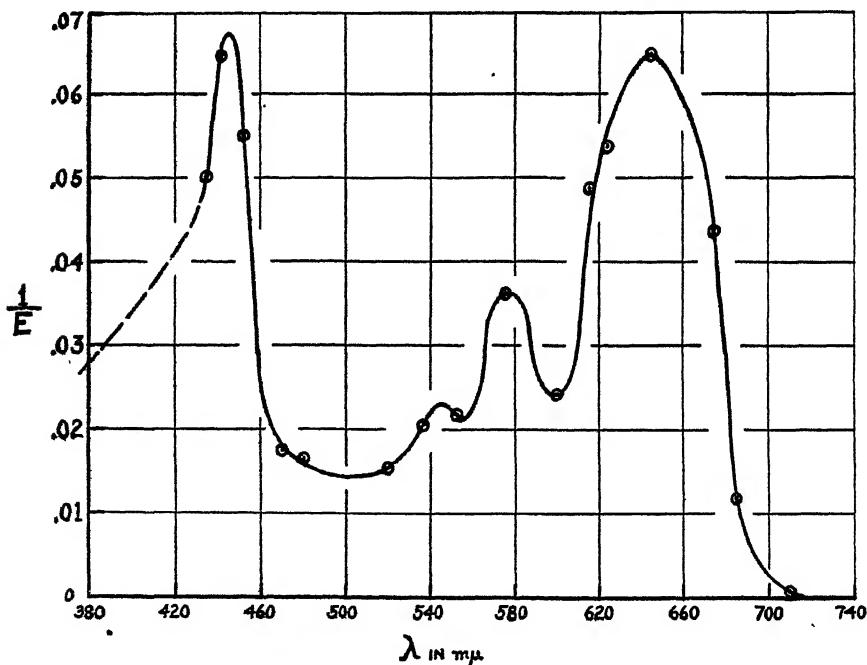


FIG. 6. First approximation effectiveness spectrum of protochlorophyll. The reciprocal of the relative amount of energy required to form a constant amount of chlorophyll is plotted against wavelength.

protochlorophyll curve does, such an approximation introduces a considerable error. The energy on one side of the center of gravity of the filter may be responsible for most of the chlorophyll formed, the other half contributing only an insignificant amount. This necessitates the calculation of a new dominant wavelength. By multiplying the reciprocal of the relative energy from the effectiveness curve at any wavelength by the energy transmitted at that particular wavelength by the filters, a new factor can be obtained for that

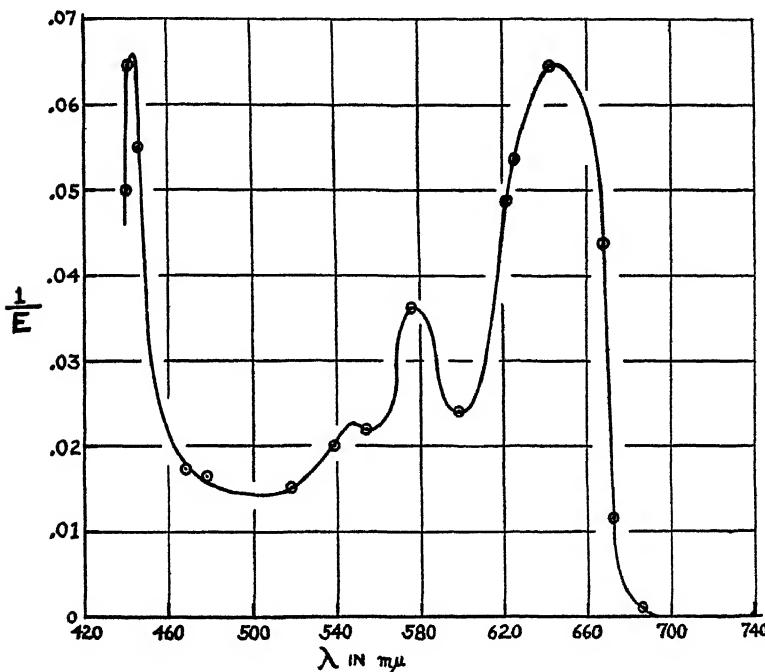


FIG. 7. Second approximation effectiveness spectrum of protochlorophyll, corrected for the error in estimating the dominant wavelength of the filter combinations.

wavelength. In this way new curves of effective energy distribution against wavelength are constructed for each filter combination and new centers of gravity determined corresponding to the dominant wavelengths. In order to apply this correction for the filters transmitting in the blue it was necessary to extrapolate the effectiveness curve beyond  $440 \text{ m}\mu$  where there are no experimentally determined points. The curve was extended into the blue in conformity with what is known of the way most porphyrins absorb there. The extrapolation is indicated as a dotted line in Fig. 6. The values of dominant wavelengths are the corrected abscissa points in the second approximation effectiveness curve plotted in Fig. 7. What has been accomplished is essen-

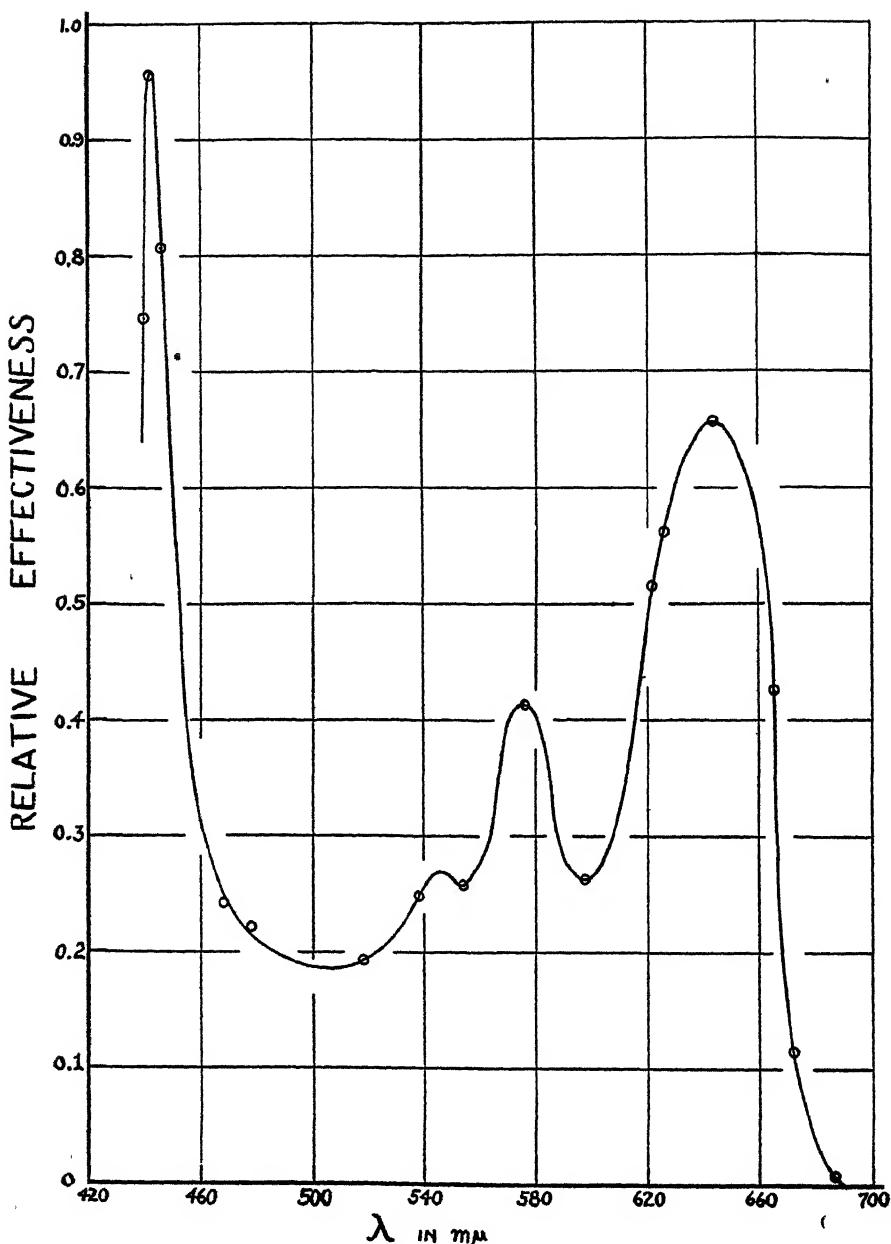


FIG. 8. Effectiveness spectrum of protochlorophyll, corrected for the quanta content of the incident light.

tially a shifting of the points on the slopes further toward the peaks resulting in a steepening of the two main absorption peaks.

A further refinement similar to the quantum correction first made on the luminosity curve for human dim vision by Dartnall and Goodeve (1937) can be applied to this curve. It is well known that light is absorbed in discrete units and that the energy of a quantum of one wavelength is different from that of another. Expressing an effectiveness curve on an energy basis does not take into account this information, since it is the quantal content of the light that one is interested in. A correction of the incident energy to an incident number of quanta can be made by multiplying the reciprocal of the relative energy at a particular wavelength by the energy of the quantum at this wavelength, which is given by Planck's formula. This value is plotted against wavelength and a new effectiveness curve results which is plotted in Fig. 8. What has been altered is the relative height of the blue and red peaks, the blue peak becoming higher than the red.

The major peaks in Fig. 8 are found at  $645 \text{ m}\mu$ ,  $445 \text{ m}\mu$ , and two minor ones at  $575$  and  $545 \text{ m}\mu$ . This effectiveness curve is related to the absorption spectrum of protochlorophyll in the condition found in the living leaf. When the pigment is extracted in organic solvents a shift in the absorption peaks is to be expected, as is the case with chlorophyll. This is borne out by examining the curves of methyl alcohol extracts of *Avena* seedlings shown in Fig. 3. The absorption found in the red portion of the spectrum of an etiolated plant extract in methyl alcohol is presumably due to protochlorophyll. There is a peak of absorption at  $630 \text{ m}\mu$  which undoubtedly corresponds to the  $645 \text{ m}\mu$  peak of the effectiveness curve. The other peaks cannot be detected in Fig. 3 since the absorption of total pigment extracts is recorded here and the carotenoids mask these protochlorophyll peaks.

#### DISCUSSION

Red light has always been considered most effective in chlorophyll formation, a finding which has been reported repeatedly by previous investigators (Lubimenko, Sayre, Rudolph, Strott, Seybold and Egle). On the whole these studies consisted in comparing the effectiveness of three broad wavelength regions, red, green, and blue. In this paper a more complete study has been presented of the relative effectiveness of various wavelengths in forming chlorophyll, the visible spectrum being divided into sixteen portions. It has been possible to show that both blue light and red light are highly active but blue light of dominant wavelength  $440 \text{ m}\mu$  is most effective in forming chlorophyll, a result not previously reported.

In the introduction to this paper several criticisms of the earlier work have been discussed, any one of which might account for the divergent result obtained here. First, the amount of chlorophyll formed for a constant duration

of light stimulus was compared for different wavelengths in the early work instead of the relative energy necessary for a constant amount of chlorophyll formed. Secondly, the duration of the light was varied as a means of controlling the total relative energy, instead of varying the total energy directly and holding the duration of the stimulus constant. And thirdly, the possible screening effect of the carotenoids in the blue region of the spectrum where they absorb heavily was not evaluated.

There is a fourth reason for the discrepancy in results, however, which offers the most likely explanation. If the curve in Fig. 8 is studied closely one can see that though the blue peak is higher than the red one, it is much narrower. Consequently, if the relative effectiveness of three broad spectral regions of *equal energy* is studied as was done by the previous workers, the result they obtained becomes understandable. The "effective" absorption of these three regions, red, green, and blue can be evaluated by multiplying the absorption spectrum of protochlorophyll by the energy distribution of the filter combinations these workers used. This product will be small for the blue end of the spectrum because the protochlorophyll absorbs only in a narrow spectral region, and largest in the red where the pigment absorption is broad and continuous over a relatively large spectral region. The result of such an investigation would be that red light is most effective in forming chlorophyll and blue light least since this rough method gives the total area of absorption, not the position of the peaks or their relative heights. A more detailed investigation of the effectiveness of small spectral regions as conducted here provides this additional information and shows that red and blue are both effective in chlorophyll formation.

The similarity but non-identity of the protochlorophyll curve to chlorophyll itself is noteworthy. Chlorophyll in aqueous digitonin extract has a peak at 675 m $\mu$  and at 445 m $\mu$ , with several minor peaks in the red (Smith, 1941). The relative heights of the blue and red peaks in the protochlorophyll spectrum are more similar to chlorophyll a than to chlorophyll b but the position of the bands closely approximates that of chlorophyll b which in ether extract has peaks at 645 and 445 m $\mu$  (Zscheile, 1935). The ratio of the blue to red peaks in the effectiveness spectrum of protochlorophyll is 1.47, in the absorption spectrum of chlorophyll a it is 1.35, and in the absorption spectrum of chlorophyll b it is 2.87. Clearly, protochlorophyll is a pigment remarkably similar to the chlorophyll pigments in whose synthesis it is involved.

The real question of whether the protochlorophyll studied here is identical with the substance investigated in the inner seed coats of the pumpkin by Noack and Kiessling and Fischer and his coworkers has yet to be considered. If the identity of the two could be established we would know that protochlorophyll differs from chlorophyll a only in lacking two hydrogens in the 7, 8 position and that the task of the light action in the synthesis of chlorophyll is to hydrogenate the protochlorophyll molecule. Since the pigment

in the pumpkin seeds has never been isolated in pure state, the absorption spectrum of the substance is unknown. Four absorption bands have been observed spectroscopically by Noack and Kiessling which are believed to be characteristic of the molecule obtained from the pumpkin seed. They are, in the order of the intensity of the bands, at 620 to 629 m $\mu$ , 560 to 576 m $\mu$ , 523 to 527 m $\mu$ , and a very dim band at 596 to 602 m $\mu$ . Allowing for the shift in absorption which one would expect for a pigment in an organic solvent as compared with its presence in the living leaf, there is an excellent correspondence between the absorption bands reported for the pumpkin seed protochlorophyll and the peaks in the *Avena* effectiveness spectrum. Only the dim 596 to 602 m $\mu$  band reported by Noack and Kiessling has no counterpart in the effectiveness spectrum curve, but this is due to an experimental limitation. In order to demonstrate this small peak it would have been necessary to determine the effectiveness at 5 m $\mu$  intervals between 580 and 620 m $\mu$ , which is not possible with filters.

The blue peak found in the effectiveness curve on the other hand has no exact counterpart in the report of the absorption properties of the pumpkin seed protochlorophyll molecule. Noack and Kiessling make the interesting statement that in dilute solution, when the extract appears greenish, there is a band in the blue "but it is unimportant" for their purposes. (In more concentrated solution the extract appears brownish.) It must be borne in mind that these authors were not dealing with pure protochlorophyll extracts and the presence of any other plant pigments would reflect itself in the absorption in the blue region of the spectrum. It was undoubtedly for this reason that the absorption there was not considered diagnostic of protochlorophyll. Until the chemical isolation of protochlorophyll can be accomplished both from the pumpkin seed and from etiolated seedlings it will not be possible to state decisively what the relation of the pumpkin seed compound is to the actual precursor of chlorophyll. But the existence of the protochlorophyll effectiveness spectrum will serve as the test of the functional significance of any such purified pigments.

An interesting insight into the morphological relation of the plastid pigments is suggested by the demonstration that the carotenoids do not filter the light from the protochlorophyll molecules. The only way for the carotenoids to be present but not to act as screens is for them to be located *behind* the protochlorophyll molecules. This would suggest that the carotenoid molecules are located inside the plastids, with the protochlorophyll molecules on the outside surface. Whether this can be applied generally to other plants would have to be experimentally determined.

#### SUMMARY

1. Although the carotenoid pigments are present in large concentration in the plastids of etiolated *Avena* seedlings as compared with protochlorophyll,

the pigment precursor of chlorophyll, it is possible to show that the carotenoids do not act as filters of the light incident on the plant in the blue region of the spectrum where they absorb heavily. This suggests that the carotenoids are located behind the protochlorophyll molecules in the plastids.

2. Since the carotenoids do not screen and light is necessary for chlorophyll formation, an effectiveness spectrum of protochlorophyll can be obtained which is the reciprocal of the light energy necessary to produce a constant amount of chlorophyll with different wavelengths. The relative effectiveness of sixteen spectral regions in forming chlorophyll was determined.

3. From the effectiveness spectrum, one can conclude that protochlorophyll is a blue-green pigment with major peaks of absorption at 445 m $\mu$ , and 645 m $\mu$ , and with smaller peaks at 575 and 545 m $\mu$ . The blue peak is sharp, narrow, and high, the red peak being broader and shorter. This differs from previous findings where the use of rougher methods indicated that red light was more effective than blue and did not give the position of the peaks of absorption or their relative heights.

4. The protochlorophyll curve is similar to but not identical with chlorophyll. The ratio of the peaks of absorption in the blue as compared to the red is very similar to chlorophyll a, but the position of the peaks resembles chlorophyll b.

5. There is an excellent correspondence between the absorption properties of this "active" protochlorophyll and what is known of the absorption of a chemically known pigment studied in impure extracts of seed coats of the Cucurbitaceae. Conclusive proof of the identity of the two substances awaits chemical purification, but the evidence here favors the view that the pumpkin seed substance, which is chemically chlorophyll a minus two hydrogens, is identical with the precursor of chlorophyll formation found in etiolated plants.

I wish to express my sincere gratitude and indebtedness to the members of the Biophysics Laboratory: to Professor Selig Hecht, for much prized friendship and stimulating teaching throughout the time I have been in his laboratory; to Dr. Simon Shlaer, for his valuable direction and suggestions given so generously, especially in connection with the research reported here; to Mr. Charles Hendley, for his friendly help in the years of our association.

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# SOME PROPERTIES OF PROTOPLASMIC GELS

## I. TENSION IN THE CHLOROPLAST OF SPIROGYRA

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Protoplasm usually contains both sol and gel.<sup>1</sup> The gel is important in determining form as well as in certain activities, including cell division.

Unfortunately we have as yet very little knowledge of the properties of protoplasmic gels.

An interesting example is found in the chloroplast of *Spirogyra*. This may undergo remarkable alterations and change from a long, spirally coiled ribbon into a short, nearly straight rod. This may occur in living cells in their natural environment, as stated by de Vries,<sup>2</sup> and may also be produced experimentally.<sup>3</sup>

Most of the observations reported in this paper were made on a *Spirogyra* with a single spiral. A normal cell<sup>3</sup> is shown in Fig. 1A. The protoplasm forms a layer not over 10 microns in thickness surrounding the large central vacuole and is held against the cellulose wall by the hydrostatic pressure of the cell sap which depends on the excess of osmotic pressure of the sap over that of the external solution.<sup>4</sup>

The chloroplast has a higher specific gravity and higher refractive index than the sap. In the dark field it appears bright, rather more so than the cellulose wall. There is little or no birefringence in the chloroplast in its normal state or when contracted by 0.01 M BaCl<sub>2</sub> or by centrifugal force.

The chloroplast appears to lie in the aqueous layer, *W*, between the outer protoplasmic non-aqueous surface layer, *X*, and the inner layer, *Y*, as in *Nitella*.<sup>5</sup> It forms a thin ribbon coiled spirally around the cell. In contracting to a straight rod it becomes detached from the peripheral protoplasm<sup>6</sup> which remains attached to the cellulose wall while the chloroplast separates off.

<sup>1</sup> The term "gel" is here used in a very liberal sense to include viscous liquid, elastic solid, and intermediate states.

<sup>2</sup> de Vries, H., *Ber. deutsch. bot. Ges.*, 1889, 7, 19.

<sup>3</sup> For a recent statement regarding this species, including a description of methods, see Osterhout, W. J. V., *J. Gen. Physiol.*, 1945-46, 29, 73.

<sup>4</sup> This is usually below 8 atmospheres.

<sup>5</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1944-45, 28, 23.

<sup>6</sup> The contraction may begin with a retraction at the ends and it may be difficult to determine how much separation occurs at that time. Regarding simultaneous change in chloroplast and protoplasm see p. 185.

Its coils gradually shorten until finally a nearly straight rod may result (Fig. 1B). Some stages in the process are shown in Fig. 2. The pyrenoids do not seem to change much during the contraction.

It was found<sup>7</sup> by the writer that contraction can be readily brought about by  $\text{BaCl}_2$  and  $\text{SrCl}_2$  without killing the cell; later Chien, working in the writer's laboratory, found  $\text{CeCl}_3$  to be still more effective.<sup>8</sup> Scarth made an extensive study of the subject, finding that contractions can be caused by a great variety of salts which are in general more effective the higher the valency of the cation.<sup>9</sup> He found that they can also be produced by alcohol, acetone, rise of temperature, and electric shocks.<sup>10</sup>

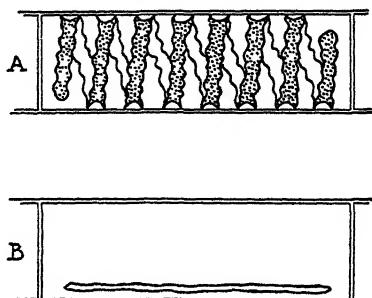


FIG. 1. Normal cell, A, before contraction; B, after contraction in 0.01 M  $\text{BaCl}_2$ . Pyrenoids omitted. Diagrammatic.

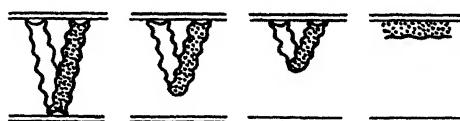


FIG. 2. Successive stages in the flattening of a single coil under the influence of 0.01 M  $\text{BaCl}_2$ . Pyrenoids omitted. Diagrammatic.

It would seem natural to suppose that these agents are the direct stimuli for contraction but further experiments indicate that their action may be indirect: these experiments involve the use of centrifugal force.<sup>11</sup>

<sup>7</sup> Osterhout, W. J. V., *Am. J. Bot.*, 1916, **3**, 481.

<sup>8</sup> Chien, S. S., *Bot. Gaz.*, 1917, **63**, 406.

<sup>9</sup> Scarth, G. W., *Tr. Roy. Soc. Canada*, Section V, 1922, **16**, 51; 1923, **17**, 137; *Quart. J. Exp. Physiol.*, 1924, **14**, 99, 115.  $\text{Ca}^{++}$  and  $\text{Zn}^{++}$  are exceptional in producing little or no effect.

<sup>10</sup> The writer finds that trypan blue acts similarly but the effect is not destroyed by ashing and it therefore seems to be due to an inorganic impurity.

<sup>11</sup> For the literature regarding centrifugal force as applied to *Spirogyra* see Weber, F., *Biochem. Z.*, 1921, **126**, 21.

*Contractions in Living Cells Due to Centrifugal Force.*—When filaments, firmly anchored to a slide by congealed drops of gelatin,<sup>12</sup> are placed in distilled water and subjected to sufficient centrifugal force (about 900 times gravity for 10 minutes) in a direction perpendicular to the long axis of the cell, the chloroplast becomes loosened from its attachment to the peripheral layer of protoplasm which remains in contact with the cellulose wall.<sup>13</sup> The chloroplast may contract to a nearly straight rod (somewhat as shown in Fig. 1B), like that obtained when certain reagents (such as  $BaCl_2$ )<sup>3</sup> are applied. This treatment does not kill the cell.

This indicates that the chloroplast is normally in a state of tension and therefore contracts as soon as it is freed from its attachment.

If only a portion of the chloroplast is set free this loosened part contracts and straightens while the rest remains coiled in spiral form (Fig. 3).

There is nothing to indicate that the process is reversible but when the chloroplast has straightened, the cell may be killed by placing it in 0.01 M oxalic acid for an hour or more and then (after washing in water) in 0.01 M

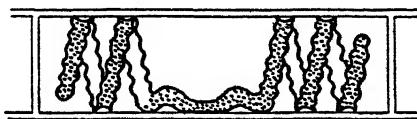


FIG. 3. Shows a cell in which the application of centrifugal force has detached 3 coils of the chloroplast and caused them to straighten. Pyrenoids omitted. Diagrammatic.

NaOH to make the chloroplast swell and elongate. It may then in some cases become somewhat longer than the cellulose wall and in consequence a few irregular zigzag bends may appear but there is no resumption of the spiral form (for the swelling of collapsed masses see p. 185).

When the centrifugal force is applied in a direction parallel to the long axis of the cell the result depends largely on the intensity. If it is not high a portion of the chloroplast may be loosened and contract, as already described. But if the intensity is great enough the entire chloroplast may quickly move to the centrifugal end of the cell before it has time to straighten and it may

<sup>12</sup> The cells studied are not in contact with the gelatin. A sheaf containing 1 to 10 filaments of *Spirogyra* was placed crosswise or lengthwise on a microscope slide and anchored by placing drops of gelatin (20 per cent) warmed to 29°C. at each end. The gelatin quickly congealed and held the filaments firmly in place. Cf. footnote 3. The slides were then placed in the centrifuge tubes.

<sup>13</sup> In some cases, especially when the centrifugal force is applied in a direction parallel to the long axis of the cell, the protoplasm may become separated from the cellulose wall in places as though it were attached to the chloroplast so firmly as to be dragged along when the chloroplast moves away from the cellulose wall.

become partly or completely coiled up at the end of the cell.<sup>14</sup> Some fusion of the different parts of the chloroplast may then occur. There may be less detachment than occurs when the centrifugal force is applied in a direction perpendicular to the long axis of the cell.

*Contractions in Dead Cells Due to Centrifugal Force.*—It might be suggested that when the chloroplast is displaced from its normal position in the layer *W* (which lies between *X* and *Y*) it comes in contact with the sap which may have a different composition<sup>15</sup> from *W*. Hence the contraction might appear to be due to a chemical stimulus.

In order to test this suggestion experiments were made on dead cells in which the composition of the sap could be made the same as in *W*.

When cells are killed<sup>16</sup> without collapse of the protoplasm by placing them for an hour or more in 0.01 M oxalic acid and then in distilled water for several hours, it is evident from observation of the speed with which acid stains penetrate the dead cell that the diffusible components of *W* and the sap soon become alike.

We then find that under the action of centrifugal force the chloroplast may be loosened from its attachment and contract as in the living cell.<sup>17</sup> (The protoplasm may in some cases become dislodged from the cellulose wall but with a space between it and the chloroplast.)

This suggests that the chloroplast contracts when it is set free from its attachment, although no chemical change in its environment may occur.

It may be added that in the presence of 2 M BaCl<sub>2</sub>, CeCl<sub>3</sub>, or LaCl<sub>3</sub> the chloroplast in dead cells may fail to loosen and contract when the usual amount of centrifugal force is applied. But these and other reagents at concentrations below 0.01 M applied to dead cells (killed in saturated solutions in water of guaiacol or of aniline or in 0.67 M formaldehyde) may cause loosening and contraction as in living cells.<sup>3</sup> (Inhibition of the effect of centrifugal force has also been noted in cells killed in 0.33 M acetic acid.)

*Contraction in Dead Cells Due to Reagents.*—In some cases cells may be killed without contraction of the chloroplast or collapse of the protoplasm. Useful reagents for this purpose are acetic acid (0.33 M),<sup>18</sup> hexylresorcinol (0.003 M),

<sup>14</sup> According to Weber (Weber, F., *Biochem. Z.*, 1921, **126**, 21) the effect of centrifugal force on living cells is more or less inhibited by ether under certain conditions.

<sup>15</sup> This is the case in *Nitella*, cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1944-45, **28**, 23.

<sup>16</sup> A convenient test of death is by plasmolysis in dilute sea water or solutions of NaCl as well as by lack of penetration of eosin, fuchsin, or trypan blue (all National Aniline Co. products).

<sup>17</sup> This is especially evident when the centrifugal force is applied in a direction perpendicular to the long axis of the cell.

<sup>18</sup> In some cases this seems to stiffen the protoplasm so that subsequent contraction due to barium is prevented.

formaldehyde (0.67 M), iodine (in the presence of KI),<sup>19</sup> and saturated solutions of aniline, guaiacol, or CCl<sub>4</sub>, but their action is somewhat uncertain. Heat (59°C.) sometimes produces this result.

In many cases the application<sup>20</sup> of 0.01 M BaCl<sub>2</sub> to such cells causes separation from the rest of the protoplasm followed by contraction of the chloroplast, as in the living cell. This is often the case when the cells have been killed in a saturated solution of guaiacol (about 0.18 M) or of aniline or in 0.67 M formaldehyde saturated with acid fuchsin.

The application of saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> may also cause contraction in such killed cells but here there is a drastic dehydration.

*Collapse*.—This is a convenient term to designate the process by which (during the death of the cell) the size of the protoplasmic mass is reduced so that its appearance resembles that shown in Fig. 4 (in many cases the reduction in size is greater than is shown in the figure). We do not know how much loss

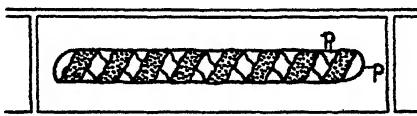


FIG. 4. Collapse of the protoplasm as the result of death, *p* is a peripheral layer of protoplasm which is normally attached to the cell wall but here is detached. Under the influence of alkali this can be made to swell up to nearly its normal dimensions with the chloroplast spirally coiled. Acid causes it to shrink and alkali may then cause it to swell again. Pyrenoids omitted. Diagrammatic.

of volume occurs in the clear protoplasm or in the chloroplast since collapse may be brought about to some extent by the escape of water from the vacuole.

Collapse may be induced by a variety of toxic substances although their action is somewhat uncertain. Good results have been obtained with 0.01 M oxalic acid,<sup>21</sup> 0.1 M acetylcholine chloride, and 2 M cellosolve. An exposure of some hours may be necessary. Saturation of the reagent with acid fuchsin may be helpful.

When such collapsed cells are placed for an hour or more in 0.01 M oxalic acid, then washed in distilled water and transferred to 0.01 M HCl for 30 minutes and then to 0.01 M NaOH the dead protoplasmic mass swells and it may regain nearly all of its normal size with the chloroplast coiled in nearly normal position. If the cell is then washed in distilled water and placed in 0.01 M HCl the whole mass shrinks and may become smaller than it was before the

<sup>19</sup> Lugol's solution diluted with 100 parts of water. The undiluted solution contains KI 6 gm., iodine 4 gm., water 100 cc.

<sup>20</sup> Usually the solution contained enough acid fuchsin to saturate it.

<sup>21</sup> Oxalic acid 0.01 M may produce collapse in some cases but in others it may kill without collapse.

application of NaOH. The transition from one state to the other can be made back and forth repeatedly in the same cell.<sup>22</sup> Each time the mass swells in 0.01 M NaOH the chloroplast resumes more or less completely its normal spiral course.<sup>23</sup> Since this does not happen when it has been separated from the clear peripheral protoplasm (p. 183) it would seem that the swelling of the clear protoplasm (which forms a hollow cylinder closed at both ends) carries the chloroplast passively with it and causes it to resume its spiral form in nearly its natural dimensions.

That an actual increase in volume occurs in the transfer from acid to alkali is seen when portions of the protoplasmic mass round up to form spheres (containing no vacuoles) the volumes of which can be readily measured. We then observe that in passing from 0.01 M HCl to 0.01 M NaOH the volume may double: this is reversible. This is also the case when the mass contains only chloroplast with no clear protoplasm.

From the relation between swelling and pH Loeb<sup>24</sup> was able to ascertain the isoelectric point of gelatin. With certain other substances, however, this could not be done and this appears to be true of the chloroplast.

With a collapsed mass of protoplasm, including the chloroplast, which has been killed in 0.01 M oxalic acid and then placed in 0.01 M HCl and then in 0.01 M NaOH we find, as already stated, that transfer to 0.01 M HCl produces marked loss in volume but if this transition is made gradually, changing the pH by small steps, there is little effect and no definite indication of an isoelectric point such as is found in gelatin.

Loeb has also shown that the isoelectric point of gelatin is indicated by its behavior toward dyes.<sup>25</sup> For example, acid fuchsin stains the gelatin more strongly below the isoelectric point (pH 4.7) than at higher pH values (after staining the dye was washed out at various pH values).

It is of interest to perform similar experiments with the chloroplast. The cell is stained on the slide for 24 hours in a 0.1 per cent solution of acid fuchsin (in 0.01 M HCl) at pH 2. The solution is drained off and the cell is then washed in 0.01 M HCl for 5 minutes after which it is examined. As the stain comes out it is found that the chloroplast retains a good deal of color: some also remains in the protoplasm but very little in the cell wall.<sup>26</sup>

<sup>22</sup> There is, however, very much less alteration in volume if the change in pH is made gradually instead of suddenly.

<sup>23</sup> To visualize the process we may imagine a crude model made by forcing into a thin-walled rubber tube enough water under pressure to dilate it to 5 times its diameter. Then a stretched rubber band is wound spirally around it and fastened at the ends. When the pressure is released and the rubber tube collapses the rubber band shortens and tends to become straighter.

<sup>24</sup> Loeb, J., Proteins and the theory of colloidal behavior, New York, International Chemical Series, McGraw-Hill Book Co., Inc., 2nd edition, 1924, 240 ff.

<sup>25</sup> Loeb,<sup>24</sup> p. 18.

<sup>26</sup> Prolonged washing removes more of the stain.

When the cell is placed in 0.1 per cent acid fuchsin in acetate buffer at pH 3.6 and washed out for 5 minutes in the same buffer containing no stain less color is retained by the cell. As we increase the pH of the acetate buffer in which the cell is stained and subsequently washed out we find that less and less color remains until above 4.7 practically no stain is retained.

Since the result with staining depends on the length of time allowed for the washing out (it was the same in all cases) another method was employed. The cell is placed in 0.1 per cent acid fuchsin and left until staining is complete and examined without removing it from the stain. It is then found that at pH 2 the chloroplast is deeply stained but the amount of staining decreases as the pH rises until above pH 4.7 very little stain is taken up by the chloroplast.<sup>27</sup>

In all these cases the cell was killed by the treatment.

#### *Experiments with Other Species of Spirogyra*

The foregoing account deals with a *Spirogyra* with one spiral which may be called species I. Additional experiments were made with a *Spirogyra*<sup>28</sup> having 2 spirals which may be called species II. When this was treated with lead acetate 0.01 M (at about pH 6) the chloroplasts contracted somewhat without becoming detached from the peripheral layer of clear protoplasm adhering to the cellulose wall (Fig. 5) so that instead of extending from end to end the chloroplasts occupied only about two-thirds of the length of the cell.<sup>29</sup> The distances between the coils shortened and the pitch increased correspondingly, suggesting a uniform contraction throughout the length of the chloroplast. It is therefore evident that under the influence of lead acetate a certain amount of retraction can occur without detachment. This result is not produced by 0.01 M lead nitrate.

To reach the stage shown in Fig. 5B usually requires about 10 minutes. If the lead acetate is promptly removed by washing in water the chloroplasts may return to their normal position in about 15 minutes: hence the process is reversible if not carried too far.

On standing longer in lead acetate irreversible changes occur. The chlo-

<sup>27</sup> If the chloroplast behaved like gelatin with an isoelectric point in the neighborhood of 4.7 we might expect addition of salts to cause contractions above the isoelectric point which would increase with the valency of the cation as stated by Scarth for the chloroplast but only if the contractions corresponded to a loss in volume and if the concentrations of the applied electrolytes were sufficiently high. Loeb has pointed out that loss of volume does not run parallel to the precipitating power of the electrolyte (Loeb,<sup>24</sup> p. 18).

<sup>28</sup> The length of the cells averaged about 120 microns and the width about 21 microns. The chloroplast was about 5 microns wide and usually showed 2 or 3 complete turns with pyrenoids at regular intervals. A "typical" nucleus was not observed but no attempt was made to demonstrate a nucleus by staining.

<sup>29</sup> This does not apply to species I, where 0.01 M lead acetate and 0.01 M lead nitrate act like 0.01 M barium chloride.

roplast becomes detached from the peripheral layer of protoplasm adhering to the cellulose wall and this is accompanied by straightening. Eventually the two chloroplasts may approach each other and form an elongated mass, the length of the mass being about one-third that of the cell and the width about half that of the cell. Inside this mass the chloroplasts appear to be more or less twisted together with some fusion.

In 0.01 M lead acetate the cells appear to live for at least 1 hour.

In 0.01 M BaCl<sub>2</sub> the chloroplasts do not contract until they become detached from the peripheral layer of protoplasm adhering to the cellulose wall. There are many cases where the ends of the chloroplasts are in the peripheral protoplasm and have not pulled away from the end walls but in the middle of the

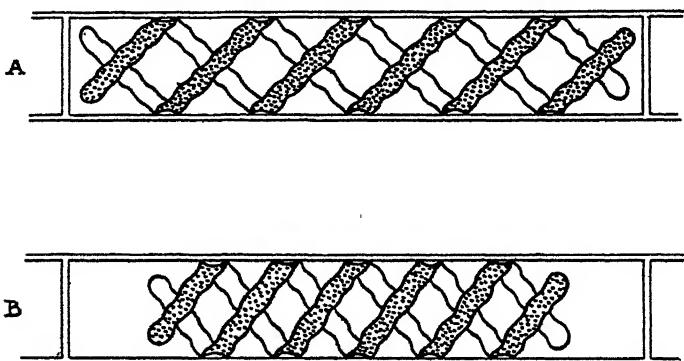


FIG. 5. *Spirogyra* with 2 spirals. A, normal. B, after exposure to 0.01 M lead acetate for 10 minutes. If the lead acetate is promptly removed the chloroplast may go back to its original state as shown at A. Pyrenoids omitted. Diagrammatic.

cell the chloroplasts are detached from the peripheral protoplasm and show contraction.

Eventually the chloroplasts become nearly straight rods running the entire length of the cell or nearly so. They lie side by side for a time but many eventually fuse more or less completely and considerable shortening may occur. As this process continues they may form a single mass occupying about two-thirds of the length of the cell.

When centrifugal force is applied perpendicularly to the long axis of normal cells the chloroplasts behave as in species I and may eventually come to lie side by side as nearly straight rods occupying most of the length of the cell.

In some cases this takes place in one chloroplast while the other retains for a time its normal position and appearance. In other cases one end of one chloroplast is at first affected while the remainder retains for a time its normal state and the second chloroplast shows no change.

Another species with 4 spirals and a typical nucleus behaves like species I in regard to BaCl<sub>2</sub> and lead acetate; with lead nitrate its behavior is similar to that with species II with lead acetate but after the preliminary retraction from the ends the process is not reversible; this retraction from the ends is followed by contraction such as is seen with BaCl<sub>2</sub>. The contraction caused by BaCl<sub>2</sub> and by centrifugal force appears to be irreversible.

It may be added that with living cells of all the species all the results hitherto described are highly variable, depending on the season of year, the length of time the cells have stood in the laboratory, and the medium in which they are kept. In many cases distilled water proved useful as a medium, in other cases a saturated solution of CaCO<sub>3</sub> or Solution A.<sup>30</sup>

#### DISCUSSION

Some important questions arise in connection with these observations. If we cannot hope to answer them at the present time we can at least mention them briefly.

1. If the chloroplast is a gel in a state of tension how does it become spirally coiled in the cell?

Evidently as the growing cell lengthens the chloroplast elongates to keep pace with it. Since the ends of the chloroplast are not in contact with the end walls the mechanism is not like that of a compressed spiral spring (which would not shorten when set free from its attachment).

In growth the cellulose wall (a hollow cylinder closed at both ends) increases in length but not in diameter (this is a matter of considerable interest which cannot be discussed here).<sup>31</sup>

The protoplasm is closely applied to the cellulose wall and is held there by the hydrostatic pressure of the cell sap and it lengthens as the cellulose wall lengthens. When the cell is killed or plasmolyzed water escapes. If the cellulose wall and the protoplasm then decrease in length the change is only slight and this applies also to the chloroplast as long as it remains imbedded in the protoplasm and consequently retains its spiral form. But when the chloroplast in the living or dead cell is set free from the clear protoplasm (by centrifugal force or otherwise) it may lose 80 per cent of its length. It would therefore seem that during growth the protoplasm stretches the chloroplast and forces it to assume a spiral course. This condition once set up may persist in the dead cell (p. 184).

The chloroplast is evidently attached along its entire length since any part which becomes free straightens at once (Fig. 3).

The course of the chloroplast may evidently be determined by the rest of the protoplasm, as happens with the longitudinal rows of chloroplasts in

<sup>30</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, 17, 87.

<sup>31</sup> There is a local increase in diameter during the process of conjugation.

*Nitella* which extend the entire length of the cell. In the youngest cells the rows are nearly straight but later both they and the protoplasm<sup>32</sup> in which they lie take a spiral course.<sup>33</sup>

It is of interest in this connection to note that in some species of *Spirogyra* with more than one chloroplast, the chloroplasts may show transitions from nearly straight to decidedly spiral. In such cases there is a closer analogy with the situation in *Nitella*.

In view of this it seems possible to suppose that the protoplasm takes a spiral course and carries the chloroplast with it so that the chloroplast may become passively stretched by the elongating protoplasm. Perhaps similar considerations apply to *Spirotaenia*<sup>34</sup> where the chloroplast may be axial in the cell with spirally twisted longitudinal ridges.

Further evidence that it is the protoplasm which determines the course of the chloroplast is seen in the fact that in species where two or more chloroplasts are present they never interfere with each other. In the species employed in these experiments a cell is occasionally seen where the chloroplast does not terminate at the end of the cell but makes a complete loop but without touching the end wall and returns for some distance on a course parallel to the coils already existing (and half way between them).

2. If the chloroplast is passively stretched by the elongating protoplasm it is not surprising that it contracts as soon as it is set free by centrifugal force or otherwise. Usually the contraction stops (for a time at least) approximately at the stage shown<sup>35</sup> in Fig. 1B. But if the cell is allowed to stand in water contraction may continue and the chloroplast may break up into a number of small masses.

If the chloroplast is passively stretched contraction may simply be a reversal of the process of stretching. Perhaps to some extent a change from gel to sol may be involved. It has been shown in a previous paper<sup>35</sup> that, in some cases at least, there is a loss of volume. This may suggest syneresis.

It may be added that when toxic agents cause collapse of the entire protoplasmic mass (p. 185) the protoplasm may acquire a granular or "coagulated" appearance which suggests precipitation.

3. How is the chloroplast held in place and prevented from contracting? Our knowledge of the forces controlling adhesion is too limited to permit analysis of this at present. Evidently the contractile power of the chloro-

<sup>32</sup> The spiral course of the protoplasm is shown by the "white line" in the protoplasm. Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1944-45, **28**, 17.

<sup>33</sup> The direction of the spiral is that of a carpenter's wood screw: in the *Spirogyra* here employed the direction of the spiral is opposite to this.

<sup>34</sup> Cf. Smith, G. M., *Fresh-water algae of the United States*, New York, McGraw-Hill Book Co., Inc., first edition, 1933, 565.

<sup>35</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1945-46, **29**, 73.

plast is very feeble. The fact that it may eventually contract to a sphere in some cases, as stated by Scarth, suggests that it may at times act as a viscous fluid.

Since it comes away from the protoplasm with a sharply defined boundary it must have a surface layer which is immiscible with water: this might be solid or liquid. Perhaps the contractile power resides chiefly or entirely in this surface layer.<sup>36</sup> When the chloroplast contracts to a sphere the surface must become very much smaller (in some cases it must shrink to about 20 per cent of its normal extent).

It may be noted that most of the reagents which set the chloroplast free so that it can contract are inorganic and according to Scarth are in general the more effective the greater the valency of the cation. It might be suggested that in addition to setting the chloroplast free they also serve as direct stimuli for contraction but in that case we must explain why the chloroplast contracts when set free by centrifugal force alone in both living and dead cells.

It is possible that the contraction may proceed differently in different cases. Scarth speaks of more than one type of contraction.

4. It would be of great interest to know what plant and animal cells contain gels under tension and what functions they perform.

In many cases such gels must play an important part in determining the shape of the cell as, for example, in the protozoa. Tension must play a part in determining the shape of many chloroplasts and of the vacuole in *Drosera* which undergoes irregular change in shape during the process of digestion.<sup>37</sup>

Certain features of cell and nuclear division suggest that gels under tension play an important part in these processes. In *Spirogyra* the cell is cut in two after nuclear division by the growth of a ring of gel or of a solid which starts at the periphery and grows until it divides the cell into two halves.

It would seem that many of the problems involved in the study of protoplasmic gels must depend for their solution upon knowledge of the properties of molecules consisting of long chains. Such studies are now being made, especially in connection with polymers and we may hope for rapid progress in this field.

It seems appropriate to say, in conclusion, that killing the protoplasm is a good way to reduce the number of variables which make the study of life processes difficult, provided that the important variables persist after death. In the present instance this appears to be the case since the chloroplast after

<sup>36</sup> The behavior recalls that of the protoplasmic strands in certain plant hairs which may rupture and slowly contract (these strands show protoplasmic motion and must be fluid with a firm surface layer). Cf. Küster, E., Die Pflanzenzelle, Jena, Gustav Fischer, 1935, Figs. 2 and 3, pp. 8 and 9.

<sup>37</sup> Lloyd, F. E., The carnivorous plants, Waltham, Massachusetts, Chronica Botanica Co., 1942.

the death of the cell continues to react to certain physical and chemical agents in much the same manner as before. We can then control the composition of the solution surrounding it as we cannot in the living cell.

#### SUMMARY

The chloroplast of *Spirogyra* is a long, spirally coiled ribbon which may contract to form a short, nearly straight rod. This happens under natural conditions and it can also be produced by a variety of inorganic salts and by some organic substances.

It also occurs when the chloroplast is freed by centrifugal force from the clear peripheral protoplasm which is in contact with the cellulose wall. It would therefore seem that the chloroplast may be passively stretched by the action of the clear protoplasm and hence it contracts as soon as it is set free. This contraction happens in dead as well as in living cells.

It would be of much interest to know how the protoplasm brings about the coiling of the chloroplast and how the chloroplast is set free by various reagents. Presumably they must penetrate the living protoplasm to produce the effects described.

In one species partial contraction without detachment from the peripheral protoplasm can be brought about by lead acetate. This is reversible. Lead nitrate does not produce this result.

The attack upon the problem is greatly facilitated by the study of dead cells. Thereby we reduce the number of variables but the chloroplast continues to react to certain chemical and physical agents in much the same manner as in the living cell and the solution surrounding it can be controlled as is not possible in the living cell.

We must await further investigation to learn what plant and animal cells contain gels under tension and what functions they perform.

# SENSITIZATION OF CELLS TO HEAT BY VISIBLE LIGHT IN PRESENCE OF PHOTODYNAMIC DYES\*

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While it has been shown previously that ultraviolet light sensitizes cells to heat (for references see Giese and Crossman, 1945) no one seems to have tried the effects of visible light in the presence of photodynamic dyes for this purpose. Since the amount of energy available in quanta of visible light is so much less than that available in the ultraviolet it is possible that no sensitization occurs. On the other hand it is well known that in the presence of photodynamic dyes enough energy of visible wavelengths is absorbed to kill (Raab, 1900). If the dye absorbing the energy can reach the sensitive molecules or can transfer the energy to them, sensitization should occur. Positive results are reported below: In other words, a sublethal dosage of visible light in the presence of photodynamic dyes, followed by a sublethal dosage of heat results in death, even though the additive effect of the two in reverse order does not kill. The implications of these results are considered in the discussion.

## *Materials and Methods*

*Paramecium caudatum* was used as the test organism and the cultures were handled in essentially the same manner described in the preceding paper (Giese and Crossman, 1945). The source of light was a 100 watt General Electric projector spot CH-4 mazda lamp. The lamp was generally run for about 30 minutes before use; by that time it had reached a fairly constant intensity as determined by photometer readings. It was used at a distance of 75 cm. from the specimens. The light was filtered through 20 cm. of water to cut out the infrared rays. To obtain only visible light Corning filter No. 3389 was used. This filter according to the information furnished by the Corning Company has a cut-off at about 410 m $\mu$  and transmits about 70 per cent from wavelength 440 m $\mu$  and 86 per cent from 510 m $\mu$  on through the visible. In some of the experiments Corning filter No. 3060 was used where it was desired to include the extreme violet end of the spectrum. This filter has a cut-off at 370 m $\mu$  and transmits about 30 per cent at 400 m $\mu$  and about 86 per cent from 510 m $\mu$  on through the visible. For determination of the intensity in absolute units a thermopile protected by neutral filters of known transmission was used. The thermopile was calibrated against U. S. Bureau of Standards Lamps. The average intensity of the light striking the experimental animals without a filter is 1390, with filter No. 3060, 1190 and with filter No. 3389, 1010 ergs/mm.<sup>2</sup>/sec.

\* This work was in part supported by funds furnished by The Rockefeller Foundation.

## EXPERIMENTAL

*1. Effects of Visible Light Alone*

Irradiation of paramecia through Corning filters No. 3060 and No. 3389 for as long as  $2\frac{1}{2}$  hours in the absence of photodynamic dyes had not the slightest visible effect on the animals nor was there any trace of sensitization to heat. This shows that even the relatively intense light is not injurious under the conditions of the experiment. These experiments serve as a check on the technique since they indicate that short wavelengths which are so effective in sensitizing to heat are not escaping through the filter and affecting the organisms. Visible light has been observed to retard division in *Blepharisma*, but in this case a reddish pigment is present in the cell (test, 1938). It is possible that if the intensity were greatly increased some injury to paramecia might occur since even killing of bacteria has been observed following huge dosages of mixed long ultraviolet and visible light (Hollaender, 1943).

*2. Effect of Visible Light in Presence of Photodynamic Dyes*

Visible light in the presence of even as low a concentration of dye as 1:200,000 (eosin or other photodynamic dyes) will kill paramecia as can be seen from Table I. After an exposure in the presence of the dye of  $\frac{1}{8}$  the lethal time there is no visible change in the activities of the paramecia. The division rate of the animals is not altered nor is a lag observed before division begins, indicating that the light has had no effect on the division mechanism of the cell. However, if such paramecia are now exposed to heat they succumb after an exposure which is short compared to that required to kill controls not exposed to light. The data for a number of dosages are summarized in Table I and Fig. 1. As the light exposure increases the thermal exposure required for killing decreases. The relationship between the two exposures is a concave curve. In this respect it resembles the results previously obtained with ultraviolet light (Giese and Crossman, 1945).

When the concentration of the dye is decreased the light dosage must be increased as shown in Fig. 1. Under these conditions less of the light needed for the sensitization is absorbed since the absorption will depend upon the number of dye molecules; i.e., the concentration available for the purpose.

Various dyes act in the same manner: thus in the fluorescein series, fluorescein, erythrosin, and eosin sensitize in the same manner but erythrosin is the most effective, fluorescein least. Even a 1:20,000 fluorescein solution sensitizes but slightly and lesser concentrations seem to be without effect except after very prolonged exposures. Eosin in 1:200,000 dilution has about the same effect as fluorescein 1:2000 as shown in Fig. 2 and in Table I. Hematoporphyrin has a striking efficiency, as shown in Fig. 2. It is possible that the efficiency is correlated with the degree or type of union between the dye molecule and the

TABLE 1  
*Sensitization to Heat by Light*

Dye	Concentration	Light dosage			Heat exposure	
		Filter	Seconds	Fraction of lethal dosage	Seconds	Fraction of lethal dosage
Eosin	1:200,000	3389	1027	0.75	126	0.26
			685	0.50	234	0.49
			342	0.25	343	0.71
			171	0.125	427	0.89
Eosin	1:20,000	3389	261	0.75	100	0.19
			174	0.50	183	0.35
			87	0.25	373	0.73
			44	0.125	505	0.99
Eosin	1:20,000	3389	248	0.75	90	0.19
			165	0.50	183	0.38
			83	0.25	333	0.69
			41	0.125	467	0.97
Eosin	1:20,000	3389	259	0.75	106	0.22
			173	0.50	189	0.39
			86	0.25	317	0.66
			43	0.125	412	0.85
Eosin	1:20,000	3060	278	0.75	151	0.29
			185	0.50	170	0.33
			93	0.25	370	0.72
			46	0.125	500	0.97
Eosin	1:20,000	None	145	0.75	63	0.13
			98	0.50	128	0.27
			49	0.25	212	0.46
			24	0.125	385	0.83
Erythrosin	1:200,000	3389	473	0.75	88	0.17
			315	0.50	222	0.43
			158	0.25	316	0.62
			79	0.125	365	0.71
Fluorescein	1:2,000	3389	1890	0.75	50	0.10
			1260	0.50	92	0.18
			630	0.25	269	0.54
			315	0.125	402	0.80
Hematoporphyrin*	1:100,000	3389	130	0.50	28	0.05
			65	0.25	65	0.12
			33	0.125	141	0.27
			16	0.062	239	0.47
None		None	6000	1.0		
			3000	0.50	67	0.14
			2460	0.41	178	0.37
			1620	0.27	345	0.71

\* Actually the concentration was less than this since not quite all of the sample dissolved.

molecules of the cell to which it unites. If the fraction of thermal lethal dose is plotted against the fraction of the light lethal dosage as in Fig. 3 the fluorescein dyes fall in one group and the hematoporphyrin falls in another, showing its much greater efficiency.

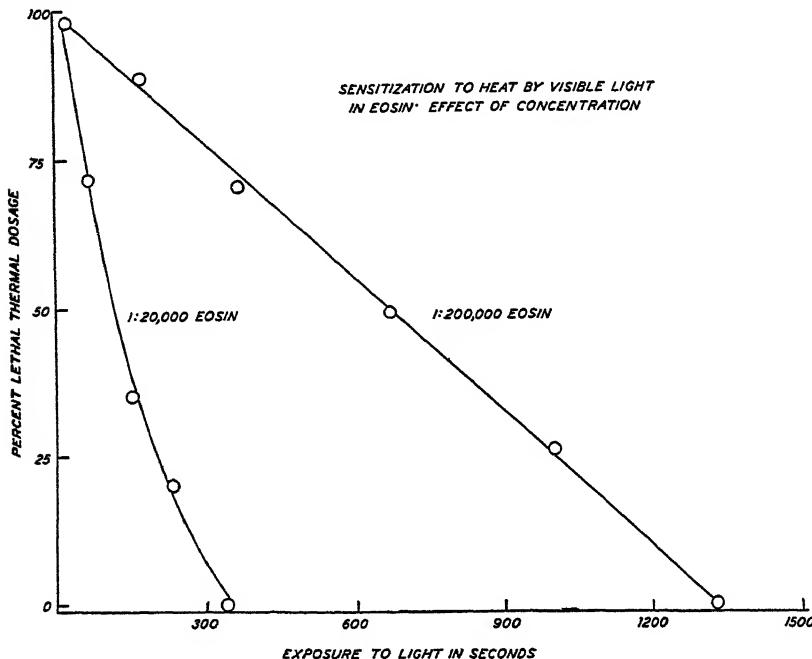


FIG. 1. Heat sensitizing effect of visible light in presence of eosin of different concentrations.

### 3. Effect of Long Ultraviolet Radiations Compared to Visible Light<sup>1</sup>

If the light is filtered only through the water cells to remove heat but not through the filters which remove long ultraviolet, it is found that prolonged radiation will kill the paramecia and sublethal dosages will sensitize them to sublethal heat exposures even without eosin. This system having 4 thicknesses of glass transmits the longer ultraviolet between about 3500 and 4000 Å in addition to the visible spectrum although no attempt was made to determine the exact limits. The exposure is about twenty times that required in the presence of eosin using visible light only (Table I and Fig. 4). This indicates that these radiations are absorbed relatively slightly. Photographs of cells in the long ultraviolet show this is indeed the case (*e.g.* Swann and del Rosario, 1932) and

<sup>1</sup> We are indebted to Miss Janet Settle for preliminary experiments with long ultraviolet radiations.

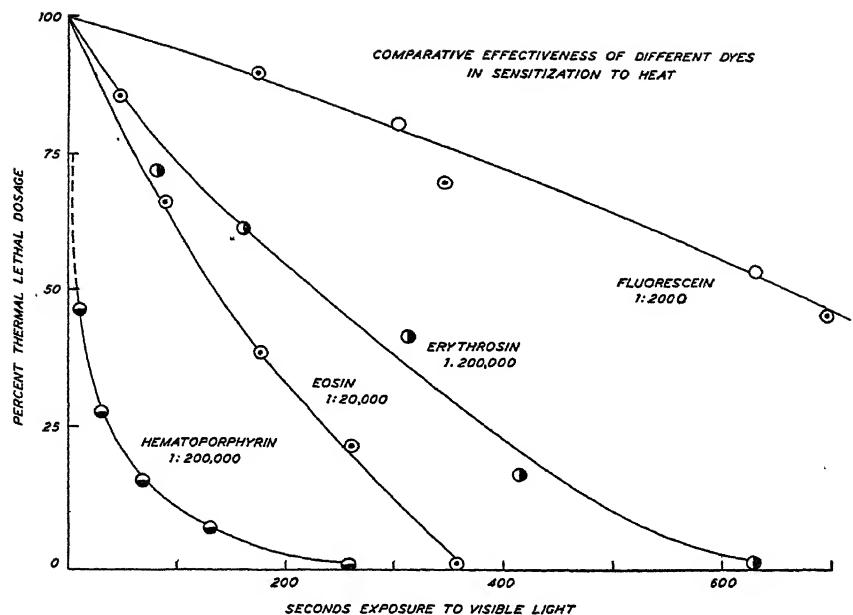


FIG. 2. Sensitization to heat by various photodynamic dyes, compared on the basis of exposure time. On the fluorescein curve the empty circles are for fluorescein 1:2000, the circles with dots are for 1:200,000 eosin added for comparison.

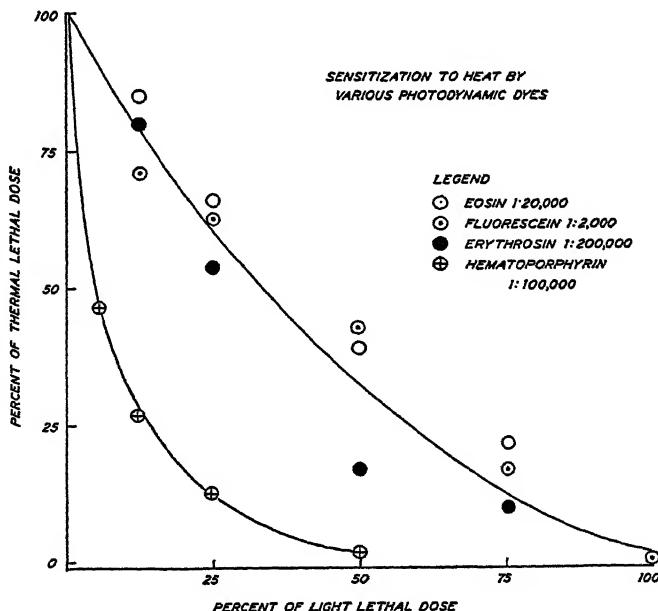


FIG. 3. Sensitization to heat by various dyes, compared on the basis of the per cent of lethal dose required for a given sensitization to heat.

spectrophotometric studies on proteins demonstrate the same for nucleoproteins and simple cytoplasmic proteins (Casperson, 1936). That apart from their inefficiency, these radiations act like the shorter radiations is not unexpected judging from their sublethal effects observed in other cases (for references see Giese, 1945, Table 2).

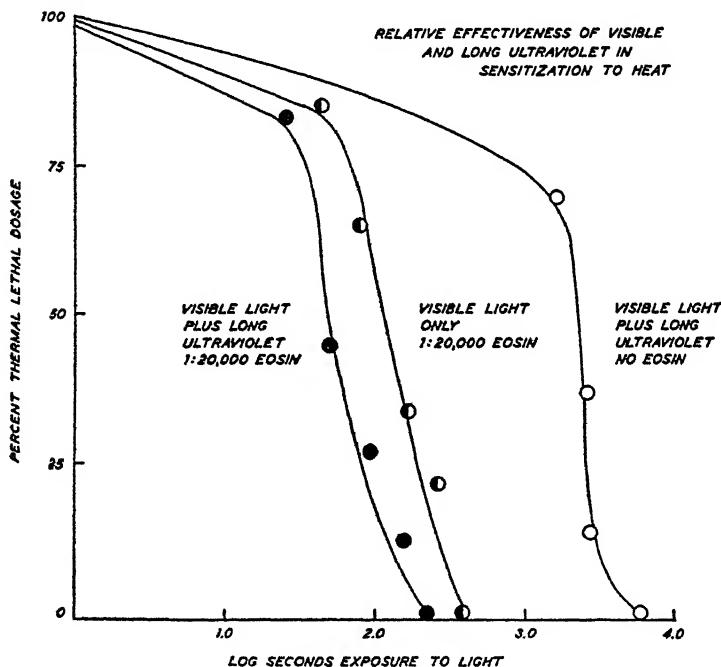


FIG. 4. Sensitization to heat by visible light in presence of eosin compared to sensitization by long ultraviolet light.

If in addition to being irradiated with all the spectrum transmitted through the water cells the paramecia are now sensitized by eosin, they are affected much more readily than when the filter is used to cut off the long ultraviolet rays. Apparently the ultraviolet radiations not only affect the cells directly but also, after absorption by eosin judging from the following facts. (1) Killing with the entire spectrum occurs in 60 per cent of the time for visible light alone, in both cases with 1:20,000 eosin. (2) No injury or sensitization occurs from visible light alone in absence of dye. (3) The injury from irradiation with the entire spectrum in the absence of dye is only  $\frac{1}{10}$  of that occurring from visible light and  $\frac{1}{3}$  of the effect from the entire spectrum in presence of the dye. The increased efficiency in (1) is much greater than can be accounted for by (3), therefore, eosin must be absorbing the long ultraviolet.

#### 4. Recovery from the Sensitization to Heat

To ascertain the nature of the sensitization effect it is necessary to determine how long the change which results in heat sensitivity is retained by the protoplasm of the cell. For this purpose the paramecia were irradiated on a given day and then tested at daily intervals until they had recovered normal sensitivity. The results are given in Fig. 5. It will be observed that about 4 days are necessary for complete recovery. Recovery occurs most rapidly during the

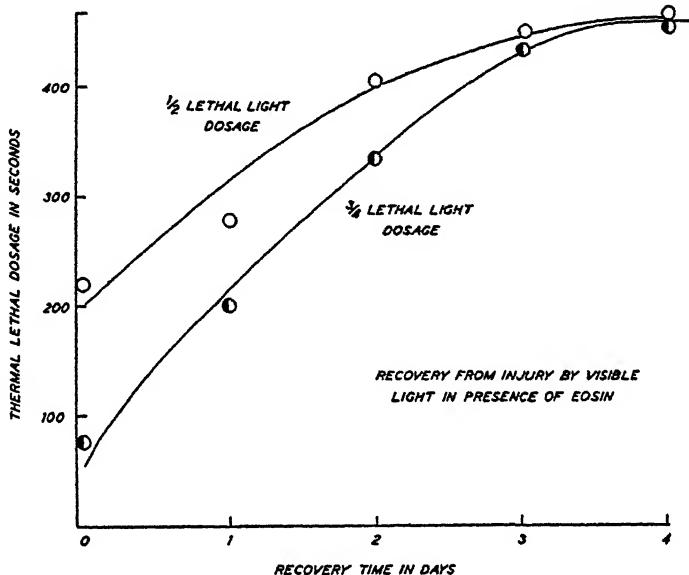


FIG. 5. Recovery from heat sensitizing effects of exposure to visible light in presence of eosin.

first day, then proceeds at almost a constant rate for the next 3 days. This shows that the effect of light is quite permanent—very much like the effect of ultraviolet radiations discussed in the preceding paper (Giese and Crossman, 1945).

#### DISCUSSION

The results described in this paper demonstrate that even visible light can sensitize cells to heat in the presence of a photodynamic dye just as long and short ultraviolet light can sensitize to heat in the absence of the dye. The mechanism by which this occurs in the visible spectrum is unknown but on the basis of various facts obtained by action of dyes upon the organism and *in vitro* and on the basis of similarities between the action of visible and ultraviolet light a possible mechanism may be outlined. Thus it is known that *in vitro* the

photodynamic dyes readily photosensitize proteins and amino acids but only slightly affect carbohydrates and lipid materials (Schmidt and Norman, 1922; Carter, 1928). The dye probably attaches itself to the protein. The protein alone is unable to absorb light but the chromophore of the dye absorbs the light and carries this energy across the linkage to a peptide or other bond. It has been demonstrated that ultraviolet light absorption results in breakage of the peptide bond (Carpenter, 1940). However, the same may not be true in the visible spectrum since there is much less energy available. Thus at 2000 Å in the ultraviolet 142,000 calories per mole are available, whereas at 4000 Å in the visible only half as much is available. Nevertheless this may be sufficient since only 48,600 calories per mole are required to disrupt the peptide bond (Pauling, 1939). The answer lies in the amount of energy lost in the transfer of the energy from its absorption by the chromophore to its action at the bond.

While the end result of the action of visible and ultraviolet rays is the same and the general mechanism may be similar the steps involved may be different. Photodynamic effects in all cases studied take place only in the presence of oxygen (Blum, 1941), whereas ultraviolet radiations act equally well in the absence of oxygen. It is thought that in photodynamic action the dye passes the energy absorbed to the molecule to which it is attached which then becomes excited. The excited molecule reacts with oxygen perhaps forming a peroxide. Such a reaction might well affect some important bonds in the molecule. Presumably some of the proteins affected in this manner might be catalytically important. So long as the culture temperature is maintained the organism is not affected and judging from the rate of recovery the important molecules are either repaired or replaced, probably the latter. When the temperature is raised the thermal agitation results in the disruption of the injured molecule. The loss of a sufficient number of such molecules results in death. In the ultraviolet part of the spectrum the chromophores which absorb the light are present in the proteins, aromatic amino acids, purines, and pyrimidines serving in this rôle. The energy absorbed is passed to other parts of the molecule and certain bonds are affected. Presumably in this case also certain important catalytic proteins are affected and so long as the culture temperature is maintained nothing happens. But if sufficient thermal agitation at a higher temperature occurs, the molecules are disrupted, leading to death.

The partial denaturation of proteins by light, visible or ultraviolet, suggested by sensitization to heat, is in agreement with the postulation of partial stepwise denaturation of proteins in a possible "A to Z" series of steps by various denaturants (Neurath, Greenstein, Putnam, and Erikson, 1944). Light may carry the protein from state A to G, let us say; heat may then carry it on to state Z.

The locus of action of ultraviolet light and photodynamic dyes may be different. Thus ultraviolet light penetrates the entire cell and is absorbed by nucleus and cytoplasm. It is probable that all proteins are involved in this

case. In photodynamic action no effects on division are observed even if just less than a lethal dosage of light is given; once the paramecia are affected to the extent that vesicles appear, they die. This suggests a superficial effect of an all-or-none type (Giese, 1943, unpublished). On the other hand Beck and Nichols (1937) have shown that although they could not demonstrate a correlation between vital staining and efficiency of photodynamic action nevertheless when the external pH is changed in such a way as may be expected to enhance penetration of the dye, it almost invariably increases its toxicity and photosensitizing powers. If this interpretation is correct the dyes may act deeper in the cell than is commonly supposed. In that case perhaps the locus of action of ultraviolet and visible light with photodynamic dyes may be similar. The slow recovery and the complete lack of any visible surface effects from sublethal dosages of light in presence of the dyes would favor the conception of a deep seated effect. Studies on the variation in the sensitization to heat by sublethal dosages of visible light in the presence of photodynamic dyes correlated with changes in pH may furnish evidence for or against this interpretation. Such experiments are in progress.

#### SUMMARY

1. Visible light of high intensity does not injure paramecia or sensitize them to heat.
2. If photodynamic dyes are added, paramecia are readily killed by visible light of high intensity and are sensitized to heat by sublethal dosages of light.
3. Cells so sensitized are killed when subjected to a sublethal exposure to heat.
4. If the light and heat are applied in the reverse order, namely, heat and then light, no ill effects are observed.
5. When the concentration of dye is reduced a larger light dosage is required.
6. Recovery from sensitization is slow, requiring about 4 days for a  $\frac{3}{4}$  lethal dosage.
7. Sublethal dosages of light in the presence of dyes do not affect the division rate even when  $\frac{3}{4}$  the lethal dosage has been used.
8. A possible explanation for the photodynamic sensitization to heat is discussed.

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## THE MECHANISM OF THE INHIBITION OF HEMOLYSIS

### V. INHIBITORY PROCESSES OCCURRING IN THE COURSE OF SIMPLE HEMOLYTIC REACTIONS

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Most investigations on the kinetics of hemolysis have been based on the idea that the lysins enter into irreversible combination with components of the red cell membrane, and imply that the lytic reaction is accompanied by a diminution in the quantity of free lysin present in the system. Attempts at expressing the velocity of hemolytic reactions accordingly contain, in one form or another, the assumption that lysis occurs when a certain amount of a cell component is transformed into a new compound as a result of a reaction with a quantity of lysin  $x$ , and so are concerned with the form of an expression of the type

$$\frac{dx}{dt} = f(c - x) \quad (1)$$

in which  $c$  is the initial concentration of lysin. The term  $(c - x)$  is the amount of free lysin in the system, and this is supposed to decrease as the reaction between lysin and cell component proceeds, so that it stops, with  $t = \infty$ , when  $x = c$ . Usually lysis is complete long before this happens, but the time for complete lysis is infinite in a system with an initial concentration of lysin equal to some particular value of  $x$  associated with complete hemolysis, and this concentration corresponds to the asymptote of the time-dilution curve. Now there are two points, essential to this theory, which are not satisfactorily settled. The first is whether there is an accumulation of lysin at the red cell surfaces, in which case the concentration of lysin to be considered in the kinetics of the systems would be some function of  $c$  rather than  $c$  itself, and the second is whether the concentration of lysin falls during the hemolytic process as the theory demands.

1. If an accumulation of lysin takes place at the red cell surfaces, it ought to be possible to demonstrate it by separating off the bulk phase immediately after the cells are added to the lysin in such concentrations as do not produce lysis in the short time necessary for the separation; the quantity of lysin present in the separated bulk phase can then be found by adding red cells and measuring the time for their complete hemolysis; *i.e.*, by a hemolytic titration. When this is done, it is found that the concentration of lysin (saponin, digitonin, and the bile salts) in the separated bulk phase is smaller than the initial concentration, and this has been interpreted as evidence for the accumulation of the

lysins at the red cell surfaces (Ponder, 1934 *a*). This explanation has now to be reviewed in the light of the possibility that inhibitory substances are present in the fluid in which the cells are suspended or are liberated immediately on the addition of the lysins. When a point like this is involved, it is clearly helpful to have an independent method, in which the measurement of the velocity of hemolytic reactions is not involved, for determining the concentration of lysin in the bulk phase. The results of applying such a method, in which the concentration of lysin is measured colorimetrically, is described in section I of this paper.

2. If the concentration of free lysin becomes less during the course of a hemolytic reaction, it ought to be possible to demonstrate the progressive decrease by separating the bulk phase after the reaction has proceeded for various lengths of time, adding red cells, and finding the time  $t$  for their complete hemolysis; this will provide, by reference to a curve showing  $t$  as a function of concentration, the concentration of lysin present at the time at which the separation was made. When this is done in the case of a reaction between saponin and stromata, the concentration of lysin in the bulk phase is indeed found to fall with time (Ponder, 1935), although in a manner different from that which is predicted on the basis of the simple reaction of expression (1). The demonstration of this progressive fall in lysin concentration once seemed to be good evidence for the correctness of the underlying idea that the hemolytic process uses up lysin, but now, in the light of what is known about the reactions between lysins and inhibitors, we have to consider the possibility that the progressive decrease in the concentration of active lysin is due to the progressive addition to the system of inhibitory substances derived from the stromata. Again one would think that the situation would be clarified if we could measure the concentration of the lysin in the bulk phase by a method which does not depend on a hemolytic reaction, such as a color reaction. The results of applying such a method are described in section II of this paper, and while this approach to the problem does not simplify the situation as much as might be hoped, it leads to a clearer understanding of the processes involved.

### *I. The Supposed Accumulation of Lysins at Red Cell Surfaces*

The question as to whether a lysin accumulates at the surfaces of red cells as soon as they are added to it, so that the effective concentration in the neighborhood of the cell surfaces is not the concentration  $c_1$  added, but a function of  $c_1$ , can be answered unequivocally in certain cases by an experiment of the following type, in which the quantity of lysin present in the bulk phase of a hemolytic system, after the addition of red cells and their subsequent rapid removal, is determined both directly by colorimetry and indirectly by a measurement of its hemolytic activity. It is necessary for this type of experiment that the lysin shall give a color reaction which is a function of its concentration

and which is not interfered with by substances such as cholesterol, proteins, etc., in the concentration in which they occur in the bulk phase, and that the concentrations of lysin used shall lie between certain limits: they must not be so great as to produce rapid lysis in step (*a*) of the procedure, nor must they be so small that they cannot produce lysis in step (*b*). These conditions are met by sodium taurocholate in dilutions of 1 in 1000 to 1 in 3000, so the procedure will be described as carried out with this lysin.

(*a*) *Preparation of Systems.*—A suspension of the thrice washed red cells of 2 ml. of human blood in 40 ml. of 1 per cent NaCl is prepared. To four tubes are added 2 ml. of sodium taurocholate in dilutions of 2.5 in 1000, 2.5 in 1500, 2.5 in 2000, and 2.5 in 3000 in phosphate buffer at pH 6.5, and 2 ml. of saline (1 per cent NaCl). The four tubes are cooled to 4°C. in ice water, and about 8 ml. of the red cell suspension is cooled at the same time. As quickly as possible, 1 ml. of the cold cell suspension is added to each of the four tubes, which are then immediately transferred to a centrifuge and spun at 5000 r.p.m. for 1 minute. The supernatant fluids, which should show no trace of either red cells or free hemoglobin, are transferred to four empty tubes without delay, the cells being discarded. The remainder of the cooled cell suspension is centrifuged at the same speed, and the supernatant fluid is removed and kept for step (*d*), below.

(*b*) *Colorimetric Estimation of Lysin.*—To four empty boiling tubes is added 0.5 ml. of sodium taurocholate diluted 1 in 1000, 1 in 1500, 1 in 2000, and 1 in 3000 in phosphate buffer, and to another four boiling tubes 0.5 ml. of the four supernatant fluids obtained in step (*a*). To each boiling tube is added 0.5 ml. of saline and 1 ml. of freshly prepared 0.9 per cent furfural in water and 6 ml. of 16 N H<sub>2</sub>SO<sub>4</sub> (Reinhold and Wilson, 1932). After the contents have been mixed, the eight tubes are placed for 8 minutes in a water bath at 70°C. They are cooled in running water, and the intensity of color in each is determined with a Lumetron photometer at 6400 Å. The furfural and acid alone give a small blank which is subtracted from the readings.<sup>1</sup>

(*c*) *Hemolytic Titration of Lysin.*—Hemolytic systems containing 1.6 ml. of the supernatant fluids from the four tubes containing red cells and lysin (step (*a*), above) are completed by adding 0.4 ml. of red cell suspension, and the times for complete hemolysis at 37°C. are determined. A standard time-dilution curve for systems containing 0.8 ml. of various dilutions of taurocholate in phosphate buffer, 0.8 ml. of saline, and 0.4 ml. of suspension is plotted at 37°C., and reference to this, together with a division by 5/4 to allow for the additional dilution of the lysin in the systems prepared in step (*a*), gives the quantities of lysin present in the supernatant fluids.

<sup>1</sup> The standards and the unknowns should be heated simultaneously, and in every experiment the values for the standards should be found and used as applying to that experiment only. The supernatant fluid from the red cell suspension gives a color value slightly in excess of that of the furfural-H<sub>2</sub>SO<sub>4</sub> blank; this is due to small amounts of chromogenic substances (not the bile salts) in the supernatant fluid, and their contribution to the total color has to be allowed for (see footnotes 2 and 3). The method is sensitive enough to detect a difference between  $c_1$  and  $c_2$  amounting to about  $\pm 2$  per cent of  $c_1$ .

(d) *Hemolytic Titration of Inhibitory Material.*—To 0.8 ml. of various dilutions of taurocholate in phosphate buffer is added 0.8 ml. of saline and 0.4 ml. of the supernatant fluid obtained from the red cell suspension in step (a). After standing at 37°C. for 60 minutes to allow the inhibitory material and the lysin to react, the hemolytic systems are completed by adding 0.4 ml. of red cell suspension, and the times for complete lysis at 37°C. observed. Comparison with a standard time-dilution curve obtained at 37°C. for systems containing 0.8 ml. of various dilutions of lysin in phosphate buffer, 1.2 ml. of saline, and 0.4 ml. of cell suspension enables the quantities of lysin rendered inactive by the inhibitory material to be calculated.

TABLE I

Initial lysin in bulk phase		Lysin in bulk phase after contact with cells				Effect of inhibitory material	
$\delta_1$	$c_1$	$c_2$ Colorimetric estimation	$c_3$ Hemolytic titration	$R$	$\Delta_3$	$c_4$	$\Delta_4$
1000	2000	1950	1270	1.57	730	1740	260
1500	1333	1330	835	1.60	498	1140	193
2000	1000	1020	695	1.44	305	900	100
3000	667	670	410	1.63	257	600	67

Values of  $c$  and of  $\Delta$  are in  $\gamma$  throughout.

The results obtained in a typical experiment with sodium taurocholate as the lysin are shown in Table I. The first two columns show the initial dilution  $\delta_1$  and corresponding concentration  $c_1$  in  $\gamma/2$  ml. The third and fourth columns show the quantity of lysin present in the bulk phase after rapid separation from the cells, and as measured by a colorimetric estimation ( $c_2$ ) and by a hemolytic titration ( $c_3$ ). The decrease in the free lysin in the bulk phase can be expressed either as  $R = c_1/c_3$ , or as  $\Delta_3 = c_1 - c_3$ . The last two columns show first the concentration of active lysin,  $c_4$ , in the systems containing inhibitory material derived from the cells (step (d), above), and then the quantity of lysin rendered inert by the inhibitory material,  $\Delta_4 = c_1 - c_4$ .

Table II shows the results of an experiment carried out in exactly the same way, but with sodium glycocholate as the lysin.

The following conclusions can be drawn from the results in Tables I and II. (1) There is no evidence of an accumulation of the lysin at the red cell surfaces, since colorimetric determinations show that  $c_2 = c_1$ , substantially. (2) The hemolytic activity of the lysin in the bulk phase, after contact with the cells, is reduced so that the value of  $c_1/c_3$  is about 1.56. This is the same result as was obtained in earlier experiments (Ponder, 1934 a), when it was interpreted as showing that the lysin accumulates at the cell surfaces, so that  $c_3 = c_1(1 - \xi)$ , where  $(1 - \xi) = 1/R$ . (3) Part of the reduction in the hemolytic activity of the bulk phase is due to the presence of inhibitory material derived

from the cells and present in the fluid in which they are suspended. In the experiment in Table I about one quarter, and in the experiment in Table II a larger fraction, of the reduction of lytic activity in the bulk phase can be accounted for in this way. (4) The remainder of the reduction in the lytic activity in the bulk phase must be due to some reaction over and above that between the lysin and the inhibitory material present in the fluid in which the cells are suspended. The possible nature of this reaction will be discussed below.

The same type of experiment can be carried out in systems containing larger numbers of cells. A suspension of washed red cells with a volume concentration as high as 0.3 is prepared, and to 2 ml. of this is added 2 ml. of lysin (taurocholate or glycocholate) in various concentrations, at 4°C. The cells are centrifuged off at

TABLE II

Initial lysin in bulk phase		Lysin in bulk phase after contact with cells			Effect of inhibitory material		
$\delta_1$	$c_1$	$c_2$ Colorimetric estimation	$c_3$ Hemolytic titration	$R$	$\Delta_2$	$c_4$	$\Delta_4$
1000	2000	2020	1280	1.56	720	1740	260
1500	1333	1350	876	1.52	457	1180	153
2000	1000	1000	644	1.55	356	910	90
3000	667	660	426	1.56	241	616	51

Values of  $c$  and of  $\Delta$  are in  $\gamma$  throughout.

once, and the concentration of bile salt in the supernatant fluids is found both colorimetrically and by a hemolytic titration. As before,  $c_2$  will be found to be substantially the same as  $c_1$ . Because of the larger number of cells in the systems, the inhibitory effects are very pronounced, and it is usually impossible to measure  $c_3$ , the concentration of active lysin in the supernatant fluids, because lysis takes so long to complete, but  $c_3$  is certainly much less than  $c_1$ , and at the same time considerably greater than  $c_4$ , the concentration of active lysin present in systems containing the supernatant fluid from the cell suspension.

The only value attached to experiments in which the volume concentration is large is that such experiments show that the bile salts do not accumulate measurably at the red cell surface even when it is very extensive; they also provide the incidental information that the bile salts do not enter unhemolyzed cells to any measurable extent.

The same type of experiment as that referred to in Tables I and II can be carried out with saponin as the lysin, but in this case the color in step (b) of the procedure is developed with the Mecke reagent (Kobert, 1887; Kofler, 1927).

This reagent is made by dissolving 1 gm. of selenious acid in 200 ml. of concentrated  $H_2SO_4$ , and is used by adding 6 ml. of the reagent to 3 ml. of saponin in saline in dilutions from about 1 in 1000 to 1 in 10,000. A brown color develops almost immediately. The intensity of color is measured with a Lumetron photometer at 5300 Å, and this must be done within 2 minutes; in practice, the measurement is made as soon as the bubbles which result from the addition of the reagent to the saponin solution have disappeared. An opalescence, followed by a cloudiness and finally by the formation of a reddish-brown precipitate, appears if one waits longer and if the saponin is sufficiently concentrated. Beer's law is followed only over a small range and in dilute solutions, as is shown by the following relation between dilution and  $\log I$ : 1 in 6,400, 9; 1 in 3,200, 18; 1 in 1,600, 32; 1 in 1,200, 46; in more concentrated solutions the opalescence and precipitate appear. The reagent is not specific for the saponins, although different saponins develop different colors with it,

TABLE III

Initial lysin in bulk phase		Lysin in bulk phase after contact with cells				Effect of inhibitory material	
$\delta_1$	$c_1$	$c_2$ Colorimetric estimation	$c_3$ Hemolytic titration	R	$\Delta_2$	$c_4$	$\Delta_4$
2000	1000	970	910	1.10	90	955	45
4000	500	505	455	1.14	45	476	24
6000	333	330	300	1.11	33	318	15
8000	250	255	220	1.14	30	244	6

Values of  $c$  and of  $\Delta$  are in  $\gamma$  throughout.

and small color values are obtained when it is added to dilute plasma or the supernatant fluids of cell or stroma suspensions; this means that suitable blanks must be set up along with the standards and unknowns.<sup>2</sup>

Table III shows the result of a typical experiment carried out in the same way as the experiments on which Tables I and II are based, except that sap-

<sup>2</sup> Because Beer's law is followed only over short ranges of dilution, it is doubtful whether the color values for these blanks can be subtracted from the color values of the unknowns in the usual way to give the final values, unless the blank values are quite small. A similar difficulty is encountered in connection with the fufural determinations. For example, in Table IV a 1 in 3000 taurocholate gives a value for  $\log I$  of 19, a 1 in 10 serum a value of 12, and the mixture of the two a value of 30; the mixture would thus seem to contain  $(30 - 12)/19$ , or 95 per cent of the lysin actually present. The general effect of subtracting the serum blanks is to make the lysin apparently present less than that really present, but this causes trouble only when the blanks have a color value of more than 10 per cent of the color value of the unknown. Even when these sources of uncertainty are taken into account, however, the colorimetric determinations in Tables I, II, III, V, and VI with fufural and the Mecke reagent are sufficiently good to make it certain that  $c_2$  is substantially the same as  $c_1$ , and that  $c_2$  is much greater than  $c_3$ .

onin is used as the lysin instead of the bile salts. The contents of Table III are arranged in the same way as those of Tables I and II.

The results of experiments of this type, with saponin as the lysin, are substantially the same as those of the experiments in which the lysin is sodium taurocholate or sodium glycocholate, except that the *R*-values tend to be smaller. The conclusions of this section accordingly are: (1) The suspension medium of a thrice washed red cell suspension contains inhibitory substances which render inert a small quantity of lysin,  $\Delta_4$ , so far as its hemolytic effect is concerned. (2) On the addition of the lysin to the cell suspension, a further quantity of lysin is rendered non-hemolytic within the short time necessary for the separation of the cells from the bulk phase of the system, and before any lysis takes place. This quantity ( $\Delta_3$ ) is several times greater than  $\Delta_4$ . (3) The colorimetric measurements show that the quantity of chromogenic material in the bulk phase, after contact with the cells, is substantially the same as that present initially,<sup>3</sup> and that no appreciable quantity of the lysin

<sup>3</sup> The observation that the concentration of taurocholate or glycocholate  $c_3$  found colorimetrically in the bulk phase, after contact with the cells, is substantially the same as the initial lysin concentration  $c_1$  although a considerable quantity of the lysin in the bulk phase is non-hemolytic, leads to the conclusion that lysin combined with the inhibitory material of the system is as chromogenic in the furfural reaction as is free lysin. This important conclusion can be arrived at in another way by showing that such inhibitory substances as cholesterol and the serum proteins do not interfere with the color reaction. This is done in Table IV, which shows the quantity of taurocholate, as a percentage of the quantity added, which is found by the furfural color reaction in the presence of cholesterol, serum albumin, and serum itself. The quantity of cholesterol added is equimolar with the quantity of taurocholate, the amount of serum added such as to give dilutions of 1 in 20, 1 in 100, and 1 in 200 in the serum-taurocholate mixture, and the amount of serum albumin added such as to give a concentration of 0.25 per cent in the albumin-taurocholate mixture.

TABLE IV

Taurocholate 1 in	Substances added				
	Serum, 1/10	Serum, 1/50	Serum, 1/100	Cholesterol, equimolar	Serum albumin, 0.25 per cent
1000	106	101	100	102	102
2000	120	100	99	102	101
3000	157	100	102	—	—
4000	—	—	—	100	98

The only important interference with the development of color occurs when serum, diluted 1 in 10, is present, and here the interference becomes greater as the concentration of taurocholate becomes less. The interference with color production is

initially present accumulates in increased concentration at the red cell surfaces.<sup>4</sup>

## *II. The Utilization of Lysin by Stromata*

The problem as to whether lysin is used up during the hemolytic process is seriously complicated by the fact that the lysis of red cells results in the liberation of inhibitory material (hemoglobin and intracellular substances). These substances are present in such large amounts in completely hemolyzed hemolytic systems that they interfere with the colorimetric determinations of both saponin and the bile salts, while if a hemolytic titration is used to determine the quantity of lysin remaining, there is difficulty in deciding whether the smaller amount of active lysin found is the result of its having been used up in the lytic reaction or to its having been rendered inert by liberated inhibitors.

To simplify the situation, the reaction between lysins and red cell ghosts (stromata) can be studied instead of that between lysins and the red cells themselves, and in this way some of the difficulties associated with the liberation of hemoglobin are avoided. In saponin-stroma systems, indeed, the effects of inhibitory material are very small, and so these systems are ideal in so far as those aspects of the problem which can be elucidated by hemolytic titrations are concerned. Unfortunately, colorimetric measurements of lysin concentrations are not satisfactory in saponin-stroma systems. Colorimetric measurements are excellent, on the other hand, in bile salt-stroma systems, although here the interpretation of the results of hemolytic titrations are rendered

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negligible, however, when serum in dilutions greater than 1 in 50, equimolar cholesterol, or 0.25 per cent serum albumin are present in the systems containing the lysin in concentrations from 1000  $\gamma$ /ml. to 250  $\gamma$ /ml. This means that taurocholate, largely combined with cholesterol or serum proteins in such a way as to be non-lytic, is as chromogenic in the furfural reaction as is hemolytically active taurocholate. Similar results are obtained with sodium glycocholate.

<sup>4</sup> Differences in the inhibitory effect of serum are observed when either the number or the kind of red cell used in a hemolytic system is varied, or when the pH is changed, and I have tried to account for this in terms of a competition for lysin between the inhibitor and the cell surfaces (Ponder, 1945 *a, b*). This competition is supposed to result from the lysin-inhibitor combination being partially reversible, and to lysin accumulating at the surfaces of different numbers and kinds of red cells to different extents. The greater the tendency (measured by  $\xi$ ) for the lysin to accumulate at the cell surfaces, the smaller the amount bound in the lysin-inhibitor combination; *i.e.*, the less the inhibition produced by a given amount of inhibitor. It now seems clear that the proper explanation for the effects of changing the number or kind of red cell, or of changing the pH of the system, does not depend upon differences in the tendency of the lysin to accumulate at the red cell surfaces. Some other explanation is required.

somewhat equivocal by the relatively large effects of inhibitory substances present in the fluid in which the stromata are suspended. Taken together, however, the observations which can be made in saponin-stroma systems and those which can be made in bile salt-stroma systems enable us to form a fairly clear picture of what happens to the lysin.

The experimental procedure is very similar to that described in section I. A suspension of the stromata of thrice washed human red cells is made by hemolyzing the cells with water, bringing the pH of the hemolysate to about 5.5 by bubbling CO<sub>2</sub> through it, and throwing down the ghosts by gentle centrifuging. The ghosts are washed, with very slow centrifuging, with saline saturated with CO<sub>2</sub>, and a suspension of stromata, equivalent to a standard red cell suspension, is prepared as already described (Ponder, 1934 a).<sup>5</sup>

To 2 ml. of various dilutions of lysin are added 2 ml. of saline, and, when the mixture has been brought to the temperature at which the experiment is to be conducted, 1 ml. of the stroma suspension, at the same temperature, is added. The stromata are either thrown down by centrifuging at high speeds immediately, and are thus separated from the bulk phase, or are allowed to react with the lysin for various times before being separated by centrifuging. In either case, the supernatant bulk phase is removed from the layer of stromata, and the concentration of lysin in it is found by a color reaction (as in step (b) of section I, furfural being used to develop the color in the case of the bile salts, and the Mecke reagent in the case of saponin), and by a hemolytic titration similar to that of step (c) of section I. Supernatant fluid from the stroma suspension is obtained at the same time as the lysin-stroma systems are centrifuged, and the inhibition produced by the inhibitory material contained in it is found by a procedure similar to that described in section I, step (d).

Results obtained in a typical experiment in which stromata react with sodium taurocholate are shown in Table V. Here  $c_1$  (the initial concentration of lysin in  $\gamma/2$  ml.),  $c_2$  (the concentration of lysin found in the supernatant fluid by the color reaction),  $\Delta_3$  (the quantity of lysin rendered inert as found by the hemolytic titration), and  $\Delta_4$  (the quantity of lysin rendered inert by the inhibitory material in the suspension medium of the stroma suspension) have the same meanings as in Tables I, II, and III. Table VI shows similar results for an experiment in which the lysin is saponin; unfortunately, it is possible in this case to measure  $c_2$  only when  $c_1$  is 200 $\gamma$ , for if  $c_1$  is smaller the color developed with the Mecke reagent is too small to allow of a satisfactory determination being made in view of the dubiety which exists as regards the blanks (see footnote 2).

<sup>5</sup> A suspension of stromata which is prepared so as to be equivalent to a standard red cell suspension as regards its effectiveness in reacting with lysin in one dilution is not exactly equivalent when reacting with lysin in other dilutions.

By taking the results contained in Tables V and VI together, the conclusions of this section are: (1) The suspension medium of a stroma suspension contains inhibitory substances which render a quantity of lysin,  $\Delta_4$ , inert so far as its hemolytic effect is concerned, and this quantity is considerably greater when the bile salts are used as lysins than when the lysin is saponin. (2) When lysin is allowed to react with a stroma suspension, a much greater quantity of lysin  $\Delta_3$  is rendered non-hemolytic, the quantity depending on the length of

TABLE V

Immediate separation				Reaction for 4 hrs.		
$c_1$	$c_2$	$\Delta_3$	$\Delta_4$	$c_2$	$\Delta_3$	$\Delta_4$
2000	1960	504	230	1919	1200	240
1333	1340	383	190	1330	910	195
1000	980	305	170	980	670	176
800	810	255	130	770	550	130

TABLE VI

Immediate separation				Reaction for 4 hrs.		
$c_1$	$c_2$	$\Delta_3$	$\Delta_4$	$c_2$	$\Delta_3$	$\Delta_4$
200	195	15	0	205	105	0
133	—	11	0	—	83	0
100	—	12	0	—	59	4
80	—	13	0	—	44	7
67	—	10	3	—	32	7

time during which the reaction takes place (see Ponder, 1935, for extensive time-concentration relations for systems containing saponin and stromata). (3) Colorimetric measurements show that the concentration of chromogenic material in the bulk phase of the lysin-stroma systems at the end of several hours of reaction is substantially the same as it was initially, before the reaction began.

#### DISCUSSION

The first observation to be accounted for is that the concentration of lysin in the bulk phase of a lysin-stroma system, as determined colorimetrically, is substantially the same as the initial concentration, even though a reaction between the lysin and the stromata has continued for hours, and though a large fraction of the lysin in the bulk phase can be shown to be inert as regards its hemolytic properties. This would be so if the lysin molecules were as chromogenic after they had reacted with the cell components as before the

reaction, and if the reaction were not to involve the lysin molecules being bound to the portion of the cell or of the ghost which remains and can be separated after hemolysis, or stromatolysis, has occurred. Such a picture of what takes place during the "fundamental reaction" of hemolysis, involving the interaction of the lysin molecules of the bulk phase with components of the cell membrane (and perhaps with more deeply placed components as well) to form lysin-stroma compounds or complexes, and the subsequent diffusion of these compounds or complexes outward into the bulk phase in which they appear as chromogenic but non-lytic, has many similarities to Schulman and Rideal's picture of what happens in the process of film penetration (Schulman and Rideal, 1937 *a, b*).<sup>6</sup> Other possibilities are that the amount of lysin utilized is so small (less than 2 per cent of  $c_1$ ) that its disappearance cannot be measured by the color reactions employed, or even that there is no utilization at all. These alternative explanations, which would call for a completely new theoretical treatment, and which lead to great difficulties in accounting for the relation between the velocity of hemolysis and the concentration of lysin, will be referred to again below.

The question which next arises is whether the disappearance of the quantity  $(\Delta_3 - \Delta_4)$ , occurring within a minute of the addition of the lysin to the cells (Tables I, II, and III) can be accounted for as being a utilization of lysin. An attempt can be made at answering this question by comparing the values of  $(\Delta_3 - \Delta_4)$  for different values of  $c_1$  with the values of  $x$ , calculated for  $t = 1$  minute from the expression which describes the experimental data best on the assumption that a reaction between lysin and cell components is really involved. The expression

$$Kt = \frac{p}{p-1} \left\{ c^{\frac{p-1}{p}} - (c-x)^{\frac{p-1}{p}} \right\} \quad (2)$$

derived from

$$dx/dt = K(c-x)^n \quad (3)$$

with  $p = 1/n$ , is one which describes the experimental values for time-dilution and percentage hemolysis curves excellently, even if empirically, and so, as a specific instance, we can calculate  $x$  for various values of  $c_1$  and for  $t = 1.0$ ,

<sup>6</sup> There seems to be no reason in the meantime why the processes of hemolysis and stromatolysis should not be thought of side by side with the process of penetration and breakdown of mixed protein-lipoid film, each kind of process throwing light on what happens in the other. Questions of mechanism are more likely to be settled by studying film penetration, and questions of kinetics by studying hemolytic and stromatolytic processes. While the kinetics of penetration have not been fully worked out, it is probable that they will require the same kind of equations as have been developed in connection with hemolytic systems, and that the same kind of difficulties, both mathematical and physical, will make their appearance once more.

using  $n = 1.5$  and  $K = 0.0012$ , these being the values which give the best fit to the time-dilution curve for sodium glycocholate at 4°C., with its asymptote at  $c_\infty = 500$ . The resulting values of  $x$  can then be compared, in Table VII, with the values of  $(\Delta_3 - \Delta_4)$  derived from Table II.

A comparison of the calculated values of  $x$  and of the experimental values of  $(\Delta_3 - \Delta_4)$  shows that some occurrence, not recognized in the simple theory on which expressions (2) and (3) are based, takes place in the hemolytic system within a short time (1 minute, and perhaps much less) of the addition of the lysin to the red cells. The nature of this occurrence is still an open question, for it might be either (a) a rapid utilization of lysin, or (b) a rapid giving-off of inhibitory material from the cell surfaces into the bulk phase of the system.<sup>7</sup> It should be emphasized, however, that it is not simply the fixation of lysin molecules which precedes film penetration, for  $c_2$ , measured colorimetrically, is substantially the same as  $c_1$ ; this means that no appreciable amount of lysin

TABLE VII

$c_1$	$x$ for $t = 1.0$	$(\Delta_3 - \Delta_4)$
2000	118	460
1333	65	304
1000	40	266
667	20	190

is fixed to the material of the cell in such a way as to be carried down when the cells are separated by centrifuging.

If these two possibilities exist as regards the values of  $(\Delta_3 - \Delta_4)$  in Tables I, II, and III, the same two possibilities exist as regards the values of  $(\Delta_3 - \Delta_4)$  in Tables V and VI. Here the large amount of lysin which disappears after a 4 hour reaction between lysin and stromata, in excess of the amounts which can clearly be accounted for by the inhibitory effects of material in the stroma suspension medium, can also be the result of (a) utilization of lysin with a diffusion of the non-lytic but chromogenic lysin-stroma compounds back into the bulk phase, or (b) the giving-off of inhibitory material from the stromata into the bulk phase and the formation of non-lytic but chromogenic compounds with the free lysin there. It would be helpful if we could make a calculation, similar to that upon which the data of Table VII are based, show-

<sup>7</sup> It is possible that the red cell as a whole represents a system which is in equilibrium with its environment, and that the addition of lysin results in a sudden transfer of material from the cell phase to the bulk phase so as to satisfy some new equilibrium conditions. One instance in which something of this sort occurs is when red cells in saline are placed between closely applied glass surfaces; the "antisphering substance," a protein, then leaves the cells to be adsorbed on the glass, and the cells become spheres (Furchtgott, 1940; Furchtgott and Ponder, 1940).

ing the quantity of lysin which would be expected to be utilized at the end of a 4 hour reaction period between lysin and stromata, and if we could show that the values of  $(\Delta_3 - \Delta_4)$  in the last two columns of Tables V and VI are greater than the calculated values of  $x$ ; the excess would then represent the result of an inhibitory process. It has been shown, however, that although the use of expressions (2) and (3) results in a good, if empirical, description of the velocity of hemolysis, the same expressions cannot be used to describe the subsequent process of stromatolysis (Ponder, 1935). As a result, we have no means of knowing to what extent a process of utilization, or alternatively a process of inhibition, is responsible for the value of  $(\Delta_3 - \Delta_4)$  in the last two columns of Tables V and VI. The explanation of the differences  $(\Delta_3 - \Delta_4)$  in the third and fourth columns of these tables presents the same problem as the explanation of the differences  $(\Delta_3 - \Delta_4)$  in Tables I, II, and III; here again we have no way of deciding whether the differences are the result of a process of utilization or of a process of inhibition.

The essence of the difficulty as regards the kinetics of hemolysis thus seems to be that we have to write

$$\frac{dx}{dt} = f(c - x - \Delta) \quad (4)$$

instead of expression (1), without being able to decide on the relative values of  $x$  and of  $\Delta$ . In one extreme case the inhibitory effects would be negligible or absent, and we would have an expression such as expression (1), which, in one form or another (e.g. expressions (2) and (3), and the expressions which result from putting  $\Delta = Bx$ , where  $B$  is a constant<sup>8</sup>) has been the basis of my treatment of the kinetics of hemolysis up to now (Ponder, 1934 b). In the other extreme case,  $x$  would be zero, and there would be no reaction involving a utilization of lysin. If this were the true state of affairs, colorimetric methods would certainly not reveal any disappearance of lysin, for there would be none, and we would have to account for the fact that hemolysis takes infinite time in a concentration  $c_\infty$  by saying that all the lysin in this concentration is rendered inert by inhibitory material. We would then have to develop some completely new theory, in which a reaction between lysin and cell components was not involved, in order to account for the experimental observation that the velocity of hemolysis is a function of lysin concentration. It may remain a possibility that no utilization of lysin occurs during the hemolytic process,

<sup>8</sup> The inhibition term  $\Delta = Bx$  or  $Bcx$  is introduced into the equation for the fundamental reaction of hemolysis in order to take account of the inhibitory effect of the hemoglobin liberated as the cells hemolyze. In a complete theory, it would be required in addition to the inhibitory terms discussed in this paper, which deals only with the events which occur on the addition of lysin and before lysis begins, and the events which occur in virtually hemoglobin-free lysin-stroma systems.

but, if so, there is no indication in the meantime as to the form which a new theory might take.<sup>9</sup>

The intermediate case of expression (4), in which neither  $x$  nor  $\Delta$  are negligible, still seems to be the one which describes the greatest number of the phenomena observed in hemolytic systems, although it must be admitted that no specific function of ( $c - x - \Delta$ ) which has been proposed so far has proved capable of describing the phenomena in their entirety. Part of the reason for this is evidently that earlier attempts at formulation did not recognize the extent to which the course of hemolytic reactions is influenced by inhibitory processes. In addition to this, it is probably an oversimplification to look upon hemolysis as the escape of fluid cell contents through a thin membrane the integrity of which is broken down by the lysin as a result of a process of film penetration, or in some other way. This picture of what happens may have to be replaced by a more complex one as our information regarding red cell structure becomes more complete.<sup>10</sup>

<sup>9</sup> The mass of the "fixed framework" of the cells in a standard hemolytic system is about 100 to 200  $\gamma$ . It is not known how much of the fixed framework corresponds to the structures which break down in the process of hemolysis, but lysis by quantities of lysin negligible in comparison to the asymptotic concentrations would have to be the result of changes initially occurring in quite a small fraction of the framework. Once initiated, the effects might of course spread to adjacent parts of the structures involved. More specifically, assuming lysis to be the result of a breakdown of a cell membrane,  $1\gamma$  of lysin or less would have to be able to destroy the integrity of membranes with a minimum mass of  $30\gamma$ . This is certainly a possibility, for the cell surfaces may break down in spots (Ponder, 1941), or each  $\gamma$  of lysin may be able to produce disorganization of a mass of material much greater than itself, whether this material is present as a membrane, or as an intracellular structure to which hemoglobin is attached.

<sup>10</sup> When the rat red cell changes from its normal form to its paracrystalline form (Ponder, 1945 c), the amount of saponin or of digitonin required to hemolyze it increases several fold, probably because more than the breaking down of a thin membrane is required for the liberation of the hemoglobin. The degree of complexity of the expressions required to describe the process will depend, of course, on the complexity of the red cell structure which has to be postulated; as soon as we pass from the simple model of a bag containing fluid enclosed in a paucimolecular membrane towards a model in which the hemoglobin is bound to an intracellular material and surrounded by a membrane varying in structure from point to point, we need to use equations developed on the basis of a three-dimensional geometry instead of simple expressions, such as (1) and (4), which apply to spatially homogeneous systems. This spatial element in the treatment of the kinetics may turn out to be necessary; for example, if disorganization of a hemoglobin-stromatin complex in the cell interior were a necessary part of the hemolytic process, a lysin would act more effectively when present in sufficient concentration to enable it to enter the cell from all over the surface than when it could enter the cell only through holes resulting from

## SUMMARY

This paper is concerned with hemolytic systems containing sodium taurocholate, sodium glycocholate, or saponin, and either human red cells or the ghosts (stromata) of human red cells. The lysins are allowed to react with the cells for a short time (1 minute or less), and with the ghosts for a long time (4 hours), and the quantity of lysin remaining in the bulk phase, after the removal of the cells or of the ghosts, is found by (a) colorimetric methods, and (b) methods which measure its hemolytic activity.

In the experiments in which the lysins react with the cells for a time so short that none of them is hemolyzed, it is found: (1) that the suspension medium of a cell suspension contains inhibitory substances which render a small amount of the lysin non-lytic, (2) that on the addition of the lysin to the cell suspension, a further and much larger amount of lysin is rendered non-lytic, and (3) that the quantity of chromogenic material in the bulk phase, after the lysin has been in contact with the cells and the latter have been removed, is substantially the same as that initially present. No appreciable quantity of lysin, accordingly, accumulates in increased concentration at the cell surfaces. The results of the colorimetric determinations show that the apparent disappearance of lysin from the bulk phase, once thought to be due to an accumulation of lysin at the cell surfaces, is the result either of an inhibitory process or of a sudden utilization of lysin unrecognized by existing theory.

In the experiments in which the lysins react with stromata for 4 hours, it is found: (1) that the suspension medium of a stroma suspension contains inhibitory substances which render some of the lysin non-lytic, (2) that when the lysin reacts with the stromata over a period of time, a much greater quantity of lysin is rendered non-lytic, and (3) that the concentration of chromogenic material in the bulk phase of the lysin-stroma system, after 4 hours of reaction, is substantially the same as it was initially. The observations can be accounted for by supposing that the lysin molecules are as chromogenic after reacting with the cell components as before, and by their not being bound to the cell or to the ghost, but diffusing back, combined with the components with which they have reacted, into the bulk phase. Such a process would have similarities to the process of penetration and breakdown of mixed protein-lipoid films. Because it is not possible at present to decide how much of the lysin is rendered inert because of utilization in a reaction with cell components, and how much because of the effect of inhibitory substances, difficulties and uncertainties arise in connection with the expressions which have been used to describe the kinetics of hemolysis.

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the breakdown of the surface in spots. In this case, the difference in effectiveness would probably be reflected in a decrease in a velocity constant with decreasing lysin concentration.

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## PROTEOLYTIC ENZYMES

### I. THE CONTROL OF THEIR ACTIVITY

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Because enzymes are among the most important regulators of animal and plant life processes, the results of the ability to control and utilize them may well prove to be far reaching. Such control and utilization can only follow an understanding of their mode of action, and of the conditions affecting their activity.

One of the most important and best studied groups of organic catalysts is the proteolytic enzymes.

#### A. CLASSIFICATION

Proteolytic enzymes, or proteases, are those enzymes which break down protein by hydrolyzing peptide linkages. The work of Bergmann and his co-workers (1936, 1937) has demonstrated that two general classes of proteases can be differentiated, the proteinases and the peptidases.

Proteinases hydrolyze peptide linkages anywhere in the protein molecule. Usually the linkages are within the molecule, *i.e.* adjacent to another peptide bond, so that these enzymes have been called "endopeptidases." They may be further classified on the basis of animal or plant origin, extracellular or intracellular action, optimal action at neutral pH or in varying degrees of acidity, and activation or inactivation by various compounds. The extracellular proteinases include pepsin (active in acid medium), trypsin and chymotrypsin (most active in neutral or slightly alkaline medium), and some yeast, mold, and bacterial proteases. The intracellular proteinases include those of animal origin, called cathepsins (found in all tissues and organs, and most active in acid medium at pH 5 to 6), and those of plant origin, including papain (from the papaya plant, optimum pH 5 to 6), ficin (from figs), bromelin (from pineapple), most yeast and mold proteases, and most bacterial proteases. Leucoprotease, the proteolytic enzyme of white blood cells (most active at neutral pH), probably functions within as well as outside of the cells, but because its properties are similar to those of trypsin it has usually been classified with this extracellular enzyme.

Peptidases hydrolyze only peptide linkages that are adjacent to the end of

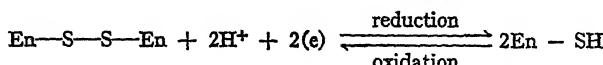
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the protein molecule, and have therefore been called "exopeptidases." They may be classified on the same bases as the proteinases, and are as widely distributed. Both proteinases and peptidases, especially the latter, have been further classified in terms of the specificity of the substrate that is hydrolyzed. Thus, carboxypeptidases hydrolyze substrates with a free carboxyl group adjacent to the linkage to be split, aminopeptidases hydrolyze substrates with a free amino group, and dipeptidases, substrates with a free carboxyl and a free amino group.

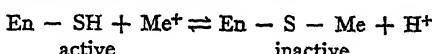
#### B. CONDITIONS AFFECTING THE ACTIVITY OF PROTEOLYTIC ENZYMES (REVIEW OF LITERATURE)

The influence of temperature and of hydrogen ion concentration on the activity of proteolytic enzymes has been carefully studied. Most of these enzymes are very active at 37°C., slightly increased in activity by short exposure to a temperature between 37 and 60°C., slightly impaired by a temperature of 65°C., and destroyed (in solution, though not in dry state) above 70°C., the necessary temperature varying inversely with the length of time of exposure. The non-specific influence of adjacent ions and molecules on the thermodynamic environment, and the specific influence of many ions, especially metallic ones, has also frequently been evaluated. Realization of the significance of oxidation-reduction systems is more recent, and while the intracellular proteinases have been intensively investigated in this regard, the important extracellular proteinases and leucoprotease have been relatively neglected.

Consideration of the importance of oxidation-reduction systems began with the discoveries that HCN (Vines, 1903, 1905), H<sub>2</sub>S (Mendel and Blood, 1910), cysteine, and HS glutathione (Grassman, von Schoenebeck, and Eibeler, 1931) increased the activity of papain and of cathepsin (Grassman *et al.*, 1930; Waldschmidt-Leitz, 1929). The physiological significance of these findings was emphasized by the demonstration (Grassman, Dyckerhoff, and von Schoenebeck, 1930) that many intracellular proteases, both plant and animal, are accompanied by sulphydryl compounds which serve as natural activators, presumably by virtue of their reducing action. A theory for the mechanism of the activation of papain by reducing agents, and of the reversible inactivation of papain by careful use of oxidizing agents such as iodine or hydrogen peroxide (Bersin, 1933, 1934) was developed by Bersin (1935) and Hellerman (1937), who suggested that the disulfide form of the enzyme (En—S—S—En) is inactive, and that reduction of disulfide groups to active sulphydryl groups activates the enzyme:



This sulphydryl mechanism has been extended by Hellerman (1937) and has been applied to many other enzymes. Further evidence for this mechanism is provided by the reversible inactivation of these enzymes (papain, cathepsin, urease) by mercaptide-forming reagents (such as benzyl mercuric chloride, *p*-tolyl mercuric chloride, cuprous oxide, etc., represented below as Me) and their reactivation by substances which decompose the mercaptides (Hellerman, 1939):



Objections to the sulphydryl theory (Bergmann and Fruton, 1941), based on varying specificities of the activators unrelated to reducing potential, and on the mechanism of activation by HCN, will not be considered at present. It is desired mainly to emphasize the significant rôle which biologically important thiol-sulphydryl compounds (glutathione, ergothioneine, cysteine, cysteine peptides, sulphydryl-containing proteins, etc.) and other oxidation-reduction compounds (*e.g.* ascorbic acid) play in the control of enzyme action. For the sake of simplicity such control will be assumed to be exerted by virtue of control of the oxidation-reduction system, though other mechanisms of action are not excluded.

Table I attempts to summarize the literature on the importance of the oxidation-reduction system in the control of the activity of many enzymes. First are listed the enzymes whose reduced form is more active than the oxidized form; then the enzymes whose oxidized form presumably is more active. Activation and inhibition have in most cases been shown to be reversible and reciprocal, particularly in that enzymes which are activated by reducing agents (especially sulphydryl compounds) are inhibited by oxidizing agents (especially inhibitors of sulphydryl groups).

As previously mentioned, the study of the influence of the oxidation-reduction system on extracellular proteinases and on leucoprotease has been relatively neglected. Grassman, Dyckerhoff, and von Schoenebeck (1930) demonstrated that cysteine,  $H_2S$ , HCN, and pyrophosphate reduced the activity of trypsin, while cystine increased its activity, but they attributed these findings to the inactivation of trypsin kinase by the reducing agents and activation by cystine, rather than to any direct effect on the enzyme. Search of the literature revealed no report on the influence of the oxidation-reduction system on leucoprotease, or on the inhibitors of trypsin and leucoprotease, including serum antiprotease.

Table II attempts to summarize the literature on substances which, in suitable concentration, have been found to influence the activity of trypsin and leucoprotease.

Because of similarity of many properties, leucoprotease and trypsin have been thought by some (Willstätter and Rohdewald, 1932) to be identical enzymes. Others (Stern, 1931) have pointed to the difference in the effect of  $Mn^{++}$  and  $Fe^{++}$  (in suitable concentrations) on these enzymes and have concluded that they are different. Comparison is difficult because crude trypsin, and leucoprotease as extracted from white blood cells, are mixtures of enzymes, and furthermore the leucoproteases of different animals may differ considerably in the kinds and amounts of enzymes present. Thus the proteinase activity of the polymorphonuclear leucocytes of carnivorous animals at pH 7 is said to be over twice as high as that of herbivorous animals (Willstätter and Rohdewald, 1932). Barnes (1940) has compared the proteinases of rabbit and cat white blood cells as follows:

	Rabbit		Cat	
	Lympho- cytes	Poly- morphs	Lympho- cytes	Poly- morphs
Proteinase active at pH 4 ("cathepsin").....	+	+	+	+
Proteinase active at pH 8 ("tryptase").....	-	-	-	+++

Parker and Franke (1917) also found no "tryptase" in rabbit polymorphs, but Weiss (1939) has reported the presence here of a "tryptase" which digests casein or gelatin

TABLE I

Proteinases activated by reducing agents
(a) Papain proteinase (79, 59, 35)
(b) Cathepsin (35, 82)
(c) Ficin and bromelin proteinase (41)
(d) Yeast proteinase (33)
(e) Proteinases of anaerobic bacteria, and of aerobic bacteria in presence of $\text{Fe}^{++}$ (85)
Peptidases activated by reducing agents
(a) Some yeast and mold dipeptidases (also activated by $\text{Zn}^{++}$ or $\text{Mn}^{++}$ ) (33)
(b) Some intestinal dipeptidases (also activated by $\text{Mn}^{++}$ ) (31)
(c) Animal intracellular ("catheptic") peptidases (29)
(d) Bacterial peptidases, especially of anaerobic bacteria (also activated by $\text{Zn}^{++}$ , $\text{Fe}^{++}$ , $\text{Pb}^{++}$ , $\text{Cu}^{++}$ , $\text{Mn}^{++}$ , $\text{Sn}^{++}$ , $\text{Cd}^{++}$ , $\text{Hg}^{++}$ ) (9)
(e) Most <i>d</i> -aminopeptidases (11)
Other enzyme systems activated by reducing agents
(a) Pneumococcal hemolysin (73)
(b) Lysozyme (60)
(c) Cerebrosidase (78)
(d) Muscle glycolysis (32)
(e) Urease (40)
(f) Serum complement (24)
(g) Stability of virus proteins (increased by reducing agents) (71, 3, 18, 61, 91)
(h) Liver pyruvate, malate, and ketoglutarate oxidase; adenosinetriphosphatase (5)
(i) <i>d</i> -amino acid, <i>L</i> -glutamic acid, and monoamine oxidase; transaminase (5)
(j) Yeast alcohol, liver choline, and bacterial glycerol oxidase (5)
(k) Liver and bacterial ( <i>E. coli</i> ) stearate, bacterial oleate, heart $\beta$ -hydroxybutyrate oxidase; ? pancreatic lipase (5)
(l) Acetylcholine esterase; ? serum mono- <i>n</i> -butyryl and liver esterase (5)
Proteinases inhibited by reducing agents
(a) Trypsin (kinase said to be inhibited) (34)
(b) Proteinases of aerobic bacteria in absence of $\text{Fe}^{++}$ (85)
Peptidases inhibited by reducing agents
(a) Some yeast and mold dipeptidases (also inhibited by $\text{Mn}^{++}$ and polypeptidases (33, 10))
(b) Intestinal leucine aminopeptidase (33), effect counteracted by $\text{Mn}^{++}$ and $\text{Mg}^{++}$ (9)
(c) Papain peptidase (12)
Other enzyme systems inhibited by reducing agents
(a) Protein synthesis by papain (80, not confirmed in 77)

at pH 8. Human polymorphs (obtained from the blood of myeloid leukemia patients) have been found to contain proteinase most active at pH 7 to 8, other proteinase most active at pH 4, and peptidase most active at pH 7 to 8 (Husfeldt, 1931).

No discussion of the inhibition of proteolytic enzymes would be inclusive without

TABLE II

## Substances which have been found to inhibit the activity of trypsin

- (a) Serum and plasma (44, repeatedly confirmed)
- (b) Charcoal (39)
- (c) Unsaturated fatty acids (49)
- (d) Tryptic digests of proteins (7, 47)
- (e) Pancreatic trypsin inhibitor (crystallized—63)
- (f) Serum trypsin inhibitor (crystallized—72)
- (g) Egg white antitrypsin (said to act by inhibiting trypsin kinase—2)
- (h) Cysteine, H<sub>2</sub>S, HCN, and pyrophosphate (said to act by inhibiting trypsin kinase—34)
- (i) Hydrogen ion concentration other than the optimum of pH = 8 (37)
- (j) Alcohol, formaldehyde, thymol, chloroform, toluol, some alkaloids, e.g. hordenine sulfate (26)
- (k) Glycerol (81)
- (l) Triglycerides, sugar (concentrated), asparagine, glutamic acid (27)
- (m) Glycylglycine, leucylglycine, and alanylglycine (84)
- (n) F<sup>—</sup>, Cl<sup>—</sup> > I<sup>—</sup> > Br<sup>—</sup>; NH<sub>4</sub><sup>+</sup> > K<sup>+</sup> > Na<sup>+</sup> (21)
- (o) Cu<sup>++</sup>, Zn<sup>++</sup> (76)
- (p) Mn<sup>++</sup> (89)
- (q) Hg<sup>++</sup> (25)
- (r) Lecithin (75)
- (s) Heparin (45)
- (t) Quinine salts (55) (act by changing pH, according to 74)
- (u) Urea salts (74)
- (v) Germanin (Bayer 205) (8)
- (w) Azodyes (56, 68)
- (x) Acridine dyes (trypafiaflavin, rivanol) and Congo red (6)
- (y) X-rays and ultraviolet light (19)
- (z) Bacteria (53)

## Substances which have been found to increase the activity of trypsin

- (a) Cystine (said to act by activating trypsin kinase—34)
- (b) Asparagine, aspartic acid, glutamic acid, cysteine, and CN<sup>—</sup> (accelerate the first stage of hydrolysis, according to 27)
- (c) Fe<sup>++</sup>, Se<sup>++</sup> (slightly) (89)
- (d) Ca<sup>++</sup>, Mg<sup>++</sup> (22)
- (e) Ag<sup>+</sup> (76)
- (f) Primary phosphate (28)

## Substances which have been found to inhibit the activity of leucoprotease

- (a) Serum and plasma (65, repeatedly confirmed)
- (b) Tryptic digests of proteins (according to 69)
- (c) Unsaturated fatty acids (50)
- (d) Hydrogen ion concentration other than the optimum of pH = 7 (76)
- (e) Fe<sup>++</sup> (89)
- (f) Quinine salts (55) (act by changing pH according to 74)
- (g) Phosphatide of tubercle bacillus (70)

## Substances which have been found to increase the activity of leucoprotease

- (a) Mn<sup>++</sup>, Se<sup>++</sup> (89)

reference to serum antiprotease. Mammalian and bird serum and plasma have long been known to strongly inhibit many animal and plant proteases, including those of pancreas (trypsin), yeast, leucocytes (leucoprotease), and tissue cells (cathepsin) (Jochman, 1908). Also present in the serum are small amounts of proteases active at neutral pH and in acid medium (Opie and Barker, 1908), but these are neutralized by a considerable excess of serum antiprotease. The nature, and the reasons for the physiological and pathological variation of the antiproteolytic activity of the serum have been the subject of much dispute. Evidence has been presented (Grob, 1943) that products of protein hydrolysis (probably one or more polypeptides) formed in the intestine and parenterally are an important factor contributing to this activity of the serum. Elucidation of the mode of action of the antiproteolytic activity of the serum would throw considerable light on conditions affecting the activity of such important proteolytic enzymes as leucoprotease and trypsin.

#### C. THE CONTROL OF THE ACTIVITY OF TRYPSIN, LEUCOPROTEASE, AND SERUM ANTIPROTEASE (EXPERIMENTAL DATA)

##### *1. Determination of Proteolytic Activity*

Proteolytic and antiproteolytic activity were determined by following the digestion of casein nephelometrically by modification of the method of Wunderly (1936). Digest mixtures were prepared by adding the following reagents in the order given:

- (a) 0 to 2 cc. of the solution to be tested for influence on protease action,
- (b) 3.5 cc. of M/15  $\text{KH}_2\text{PO}_4 - \text{K}_2\text{HPO}_4$  buffer (pH 7.5),
- (c) 0 to 2 cc. of 0.85 per cent NaCl,
- (d) 1 cc. of enzyme solution,
- (e) 3.5 cc. of 0.5 per cent casein solution in 0.006 N NaOH (pH 10.4),
- (f) 5 drops of toluol (did not influence digestion, and prevented bacterial growth if tubes were stoppered).

The total volume of each digest mixture was 10 cc. Ten minutes were allowed to elapse between the addition of the enzyme and the casein solution, thus allowing exposure of enzyme to inhibitor for an appreciable time interval before addition of substrate. Immediately after the addition of the substrate and mixing, 2 cc. of the mixture were removed for determination of pH (by Beckman glass electrode), and another 2 cc. were removed, added to 2 cc. of 25 per cent HCl and 1 cc. of 20 per cent sulfosalicylic acid, and the turbidity of the resulting suspension of undigested casein determined with the aid of a photoelectric nephelometer. Similar determinations of the concentration of undigested casein were again made after an interval of digestion at 37°C. Calibration of the nephelometer, *i.e.* translation of turbidity measurements into corresponding concentrations of casein, was possible, since known concentrations of casein were found to transmit light in accordance with Lambert-Beer's law, the concentration of turbid particles being proportional to the logarithm of the per cent of incident monochromatic light transmitted. A green filter which transmitted light of 540 millimicrons wave length gave the greatest range of scale readings. The logarithm of the per cent of incident monochromatic light absorbed ( $R$ ) was translated into corresponding concentration of casein ( $C$ ) by means of the calibration factor

( $F = C/R$ ). Concentrations of solution ( $a$ ) in the digest mixture which caused the pH to vary outside of the range 7.4 to 7.9 are not included in the tables. The pH of the digest mixture in the absence of solution ( $a$ ) was 7.8.

The concentration of crude trypsin in the digest mixture was 0.01 mg./cc., of crude papain (partly activated by dissolving in water containing a few milligrams of cysteine which was then removed by dialysis) 0.1 mg./cc., and of crystalline trypsin 0.005 mg./cc. The concentration of leucoprotease (cat) that was used produced approximately the same degree of casein proteolysis in 17 hours as was produced in 7 hours by 0.01 mg./cc. of crude trypsin, in 17 hours by 0.1 mg./cc. of crude papain when fully activated, and in 1 hour by 0.005 mg./cc. of crystalline trypsin. (These were the digestion times, at 37°C., used throughout the experiment.)

The initial concentration of casein in each digest mixture was 1.75 mg./cc. The concentration of casein after partial digestion was determined nephelometrically, as described above. Subtraction disclosed the concentration of casein that was digested by the enzyme. This enabled calculation of the concentration of free, *i.e.* active, enzyme with the aid of Schutz's "law":

$$X K\sqrt{T} \text{ (at constant incubation time)}$$

where  $X$  = concentration of protein digested,

$T$  = concentration of free (*i.e.* active) enzyme,

$K$  = a constant which is determined by digesting casein with known concentrations of each enzyme for the time intervals used experimentally, (*e.g.* 0.01 mg./cc. of crude trypsin digested 0.306 mg./cc. of casein in the standard time of 7 hours so that  $K = 3.06$  for these conditions).

Since the initial concentration of protease ( $E$ ) in each case was 0.01 mg./cc. of crude trypsin, 0.005 mg./cc. of crystalline trypsin, 0.1 mg./cc. of papain, and an equivalent in leucoprotease activity, the per cent of  $E$  that was free (*i.e.* active) in the presence of solution ( $a$ ) is equal (for crude trypsin) to  $\frac{T}{0.01} \times 100$  per cent =  $T \times 10^4$  per cent,

for crystalline trypsin  $\frac{T}{0.005} \times 100$  per cent =  $2 \times T \times 10^4$  per cent, and for papain

$\frac{T}{0.1} \times 100$  per cent =  $T \times 10^3$  per cent.  $T$  was calculated for each digest mixture

(and expressed in equivalent weight of trypsin in the case of leucoprotease), and the results are recorded in the tables as "the per cent of enzyme activity."

Wherever serum or plasma was used in these experiments the source was human.

## 2. Preparation of Leucoprotease

The proteolytic enzymes that were studied by this method were crude trypsin (Fairchild, beef), crystalline trypsin, lyo-leucoprotease, desmo-leucoprotease, and crude papain. Sufficient crystalline trypsin was available for only part of the experiments. Results obtained with desmo-leucoprotease were essentially the same as those obtained with lyo-leucoprotease, so that only the latter are recorded.

Polymorphonuclear leucocytes were obtained from rabbits and from cats by injecting 10 cc. of 5 per cent aleuronat and 5 per cent tragacanth into each pleural and peritoneal cavity of 8 animals (4 of each species), killing the animals after 24 hours by bleeding from the carotid artery, collecting the exudates in isotonic saline plus citrate, centrifuging, and washing the sedimented leucocytes several times with isotonic sodium chloride to thoroughly remove the supernatant fluid, which has been found (Opie, 1905, 1906) to be high in antiproteolytic activity. Smears of the sediment showed over 90 per cent polymorphonuclear leucocytes, the remainder being mononuclears and red blood cells. Leucoprotease may be obtained from the sediment by allowing the cells to autolyze, or by extraction with glycerol. The method of extraction was used, the sediment being shaken for several hours in 100 per cent glycerol and then centrifuged. The supernatant contains the lyo-leucoprotease ("free" leucoprotease) of Willstätter and Rohdewald (1932). The resulting sediment was then extracted with 60 per cent glycerol and centrifuged, the supernatant now containing the desmo-leucoprotease ("bound" leucoprotease).

Both the lyo and desmo preparations from rabbit leucocytes failed to show appreciable protease activity (on casein substrate at pH 7.8). This is confirmatory of the findings of Parker and Franke (1917) and Barnes (1940), who used other methods of preparation. Preparations from cat leucocytes were high in protease action at neutral pH, and are discussed below. It is interesting in this respect to note that skin and subcutaneous abscesses (as produced by injection of staphylococci) were found to be grossly different in rabbits and in cats. In cats (as in man) resolution, or liquefaction, of such abscesses commonly took place, presumably because of proteolysis by leucoprotease. In rabbits such liquefaction was found to be much slower and less marked, with the result that the abscess contents had a caseous, or "cheesy," appearance. It is suggested that this difference is due to the absence from rabbit polymorphonuclear leucocytes, and the presence in cat (and human) polymorphs, of protease active at neutral pH.

### 3. Substances Which Inhibited Leucoprotease and Trypsin

(a) Reducing Agents.—Cysteine, sodium thioglycollate, H—S glutathione, ascorbic acid, sodium cyanide, and hydrogen sulfide, (in concentrations indicated in Table III), were found to be strongly inhibitory of leucoprotease and trypsin. These reducing compounds (with the exception of ascorbic acid) considerably increased the activity of partly activated papain, showing the contrast in the influence of the oxidation-reduction system on these proteases.

The reducing capacity of each digest mixture was determined by its ability to decolorize a 0.0005 M solution of sodium-2,6-dichlorophenol indophenol. Charts 1 and 2 show that for each reducing agent the degree of inhibition of leucoprotease and trypsin, and the degree of activation of papain, are propor-

tional to the reducing capacity of the medium. The results also indicate that there is no relation between the reducing capacity of different reductants and

TABLE III

*The Influence of Reducing Agents on the Activity of Leucoprotease, Trypsin, and Papain*  
 (The per cent of enzyme activity is recorded. The reducing capacity is recorded as the cubic centimeters of 0.0005 M Na-2,6-dichlorophenol indophenol decolorized by 1 cc. of digest mixture.)

Compound	Concentration in digest mixture gm./100 cc.	Reducing capacity of digest mixture	Leucoprotease	Crude trypsin	Crystalline trypsin	Papain
None (control).....	—	0	100	100	100	3
Cysteine.....	0.10	5.5	0	44	41	100
“ .....	0.05	2.9	32	70		
“ .....	0.04	2.5	38	79		77
“ .....	0.02	1.3	60	90		
“ .....	0.01	0.5	72	100		47
“ .....	0.005	0.1	79			
Sodium thioglycollate.....	0.60	1.2	76			
“ “ .....	0.40	0.75	90	66	74	100
“ “ .....	0.10	0.25	95			
“ “ .....	0.04	0.10	106	100		82
Glutathione.....	0.14	0.5	53	79	83	98
“ .....	0.035	0.3	72	88		75
“ .....	0.014	0.1	105	96		2
Ascorbic acid.....	0.10	9.5	31	89	70	0
“ “ .....	0.05	4.8	44	92		0
Ascorbic acid + Fe <sup>++</sup> .....	0.10 + 0.01 Fe <sup>++</sup>	9.0	1	52	42	13
“ “ “ .....	0.05 + 0.005 Fe <sup>++</sup>	4.5	17	70		0
“ “ “ .....	0.02 + 0.002 Fe <sup>++</sup>	0.5	62	90		0
NaCN.....	0.25	0	8	28	50	106
“ .....	0.125	0	14	55		98
H <sub>2</sub> S.....	1/5 saturated		18	60	93	28
“ .....	1/50 saturated		87	106		13

their effect on the enzymes. This is no doubt due in part to differences in the reducing potential of each reductant. For instance, ascorbic acid, half reduced, has an E<sub>0</sub> of +0.06 volt at pH 7, while cysteine has an E<sub>0</sub> of -0.3 volt, so that it is not surprising that ascorbic acid has less effect on the enzymes

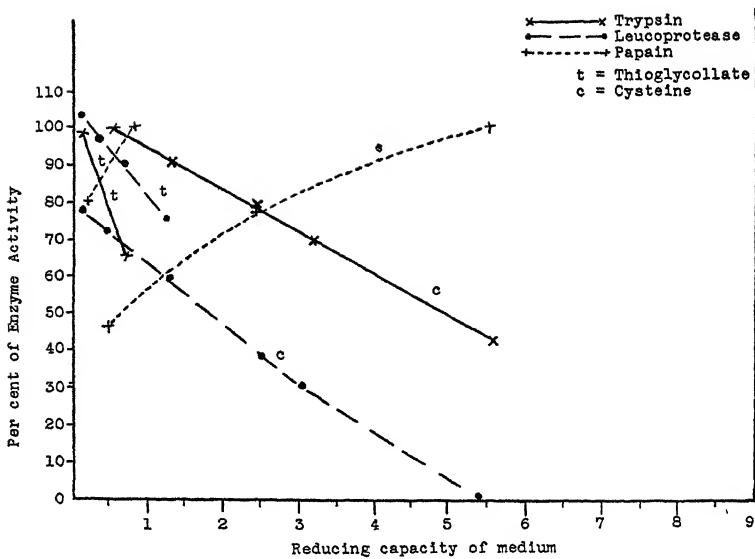


CHART 1. The influence of reducing agents (cysteine and sodium thioglycollate) on the activity of leucoprotease, crude trypsin, and papain. (The reducing capacity of the medium is recorded as the cubic centimeters of 0.0005 M Na-2,6-dichlorophenol indophenol decolorized by 1 cc. of digest mixture.)

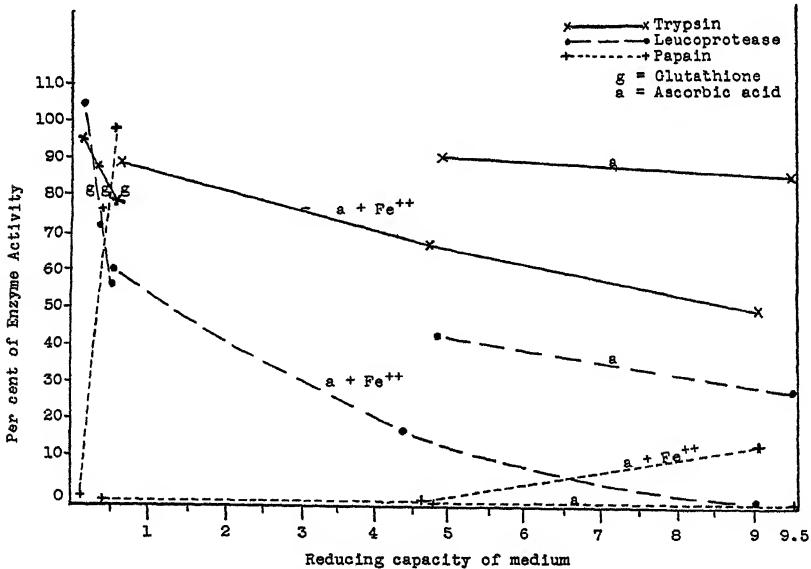


CHART 2. The influence of reducing agents (glutathione, ascorbic acid, and ascorbic acid + Fe<sup>++</sup>) on the activity of leucoprotease, crude trypsin, and papain. (The reducing capacity of the medium is recorded as the cubic centimeters of 0.0005 M Na-2,6-dichlorophenol indophenol decolorized by 1 cc. of digest mixture.)

than cysteine even when the reducing capacity of the former is higher. However, that other mechanisms may also be involved is suggested by the finding that the addition of a few milligrams of ferrous salt increased the inhibition of leucoprotease and trypsin by ascorbic acid (in the case of trypsin considerably in excess of the additive effect (see Table V)), and caused papain to be slightly activated by ascorbic acid, without changing the reducing capacity of the medium. A possible mechanism for the greater inhibition of trypsin and the beginning activation of papain is suggested by the observation (Parr, 1935) that the ferrous-ascorbic acid complex is a much more potent reducer of disulfide to thiol groups than ascorbic acid alone. Any inactive disulfide compounds in the mixture would thereby be converted into potent thiol compounds.

The contention of Grassman, Dyckerhoff, and von Schoenebeck (1930), that cysteine,  $H_2S$ , HCN, and pyrophosphate retard tryptic activity because they inhibit trypsin kinase is disproved by demonstration of the inhibition of crystalline trypsin and of leucoprotease, both of which exist in the activated state and require no kinase for their action. (Willstätter and Rohdewald, 1932, demonstrated that leucoprotease does not require kinase.)

The physiological significance of this inhibition of trypsin and leucoprotease is made evident by recalling that these enzymes probably never act in the body without the presence of appreciable concentrations of biologically important thiol-sulfhydryl reductants (glutathione, ergothionine, cysteine, cysteine peptides, sulfhydryl-containing proteins), as well as ascorbic acid. The thiol-sulfhydryl compounds were known to activate the intracellular cathepsins (just as they activate papain) but their significance as inhibitors of trypsin and leucoprotease has not been appreciated. Similarly ascorbic acid has been known to activate cathepsin and to inhibit papain (except in the presence of sufficient ferrous ions (Maschmann and Helmert, 1934) and disulfide-thiol compounds (Parr, 1935)), but its inhibition of trypsin and leucoprotease has not previously been evaluated.

(b) *p*-Aminobenzoic Acid, Sulfonamides, and Diphenyl Sulfones.—Evidence has been presented (Grob, 1943) that the growth of bacteria in the body will be more rapid, and the inhibition of sulfathiazole will be greater, when considerable protease is present and when the fluid inflammatory exudate is small and poor in antiprotease. Protease is believed to exert this effect because the products of proteolysis both accelerate bacterial growth (especially when the medium is poor in non-protein nitrogen) and inhibit sulfonamide action. Because of this physiological significance of protease, the influence on leucoprotease of the sulfonamides and related compounds was studied. Results are recorded in Table IV. They show that *p*-aminobenzoic acid and the sulfonamides tested (especially sulfathiazole) inhibited leucoprotease in physiological concentrations. Promin and a number of other diphenyl sulfones (kindly provided by Dr. Eleanor Bliss) were similarly tested, in an effort to find a

compound of greater inhibitory power that might be used to inhibit leucoprotease when desired, for instance in assisting the action of sulfonamides in purulent lesions. The sulfones that were tested inhibited leucoprotease no more than the sulfonamides, and except for promin, the sulfoxalate condensation

TABLE IV  
*The Influence of p-Aminobenzoic Acid, Sulfonamides, and Diphenyl Sulfones on Leucoprotease and Trypsin*  
 (The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture gm./100 cc.		
		Leucoprotease	Crude trypsin
Control.....	—	100	100
p-Aminobenzoic acid.....	0.040	20	97
"      ".....	0.010	90	
"      ".....	0.004	107	
Sulfathiazole.....	0.040	10	104
".....	0.020	17	
".....	0.010	25	
".....	0.004	33	
Sulfanilamide.....	0.040	18	110
".....	0.020	21	
".....	0.010	37	
".....	0.004	59	
Sulfadiazine.....	0.040	25	90
".....	0.020	40	
".....	0.010	60	
".....	0.004	87	
Sulfaguanidine.....	0.040	4	100
".....	0.020	9	
".....	0.010	22	
".....	0.004	54	

product of diphenyl sulfone, *p,p'*-diglucosaminodiphenyl sulfone, and *p,p'*-diaminodiphenyl sulfone salicylate, they have the disadvantage of being much less soluble.

The compounds that were tested inhibited (crude) trypsin slightly or not at all, so that values obtained for trypsin are for the most part omitted. It may be that smaller concentrations of trypsin would have been inhibited. (The initial concentration of trypsin in the digest mixtures was greater than that of

leucoprotease, since leucoprotease digests were incubated longer than the tryptic digests in order to reach the same extent of casein digestion.)

(c) *Heavy Metals.*—Aluminum, manganous, ferrous, mercuric, zinc, and cupric salts, in the concentrations shown, inhibited leucoprotease and trypsin

TABLE IV—*Concluded*

Substance	Concentration in digest mixture gm./100 cc.	Leucoprotease
Promin.....	0.025	74
“ .....	0.012	84
“ .....	0.002	101
Diphenyl sulfones		
<i>p</i> , <i>p'</i> -Diaminodiphenyl.....	0.020	87
<i>p</i> , <i>p'</i> -Diacylamino.....	“	76
<i>p</i> , <i>p'</i> -Diformylamino.....	“	72
<i>p</i> , <i>p'</i> -Dipropionylamino.....	“	65
<i>p</i> , <i>p'</i> -Dibutyrylamino.....	“	72
<i>p</i> -Acetoamino <i>p'</i> -amino.....	“	56
<i>p</i> -Sulfoxalate.....	“	68
<i>p</i> -Benzylideneamino <i>p'</i> -amino.....	“	65
2,4-Dinitro 4'-amino.....	“	76
<i>N</i> <sub>1</sub> Benzol <i>p</i> , <i>p'</i> -diamino.....	“	84
<i>p</i> -Propionylamino <i>p'</i> -amino.....	“	78
<i>p</i> -Butyryl <i>p'</i> -amino.....	“	92
<i>p</i> -Acetylamino <i>p'</i> -hydroxy.....	“	68
Disulfonamido.....	“	76
Diphenyl sulfone.....	“	84
<i>p</i> -Cinnamalamino <i>p'</i> -amino.....	“	74
<i>p</i> -( <i>N</i> - <i>p</i> -tolylglycyl)amino <i>p'</i> -amino.....	“	81
<i>p</i> , <i>p'</i> -Diglucosamino.....	“	74
<i>p</i> , <i>p'</i> -Diaminodiphenyl sulfone salicylate.....	“	80
<i>p</i> , <i>p'</i> -Diethanolamino.....	“	84
2,4-Dinitro 4'-acetoamino.....	“	74
<i>p</i> -Monoacetylamino .....	“	56

(Table V). Manganous and ferrous salts had a much greater inhibitory effect on leucoprotease than on trypsin.

(d) *Other Inhibitors.*—Other substances which were found to inhibit leucoprotease (and usually trypsin) are listed in Table VI. These include serum and plasma (which are equally active in the inhibition of leucoprotease and trypsin and are among the most potent inhibitors of these enzymes), thiourea, heparin, glutamic acid, tyrothrinin, ammonium salts, calcium chloride, asparagine, bile salts and acids, and (to a lesser degree) organic mercury compounds.

(e) *Polypeptide Trypsin Inhibitors.*—Polypeptide inhibitors of trypsin can be

prepared in concentrated form from a tryptic digest of casein (Hussey and Northrop, 1923). Such a concentrate was prepared, and similar concentrates were made from tryptic digests of egg albumen and serum albumin. A trypsin inhibitor preparation was also extracted (by the method of Balls and Swenson, 1934) from the thin fraction of egg white, which is believed to be a proteolytic product of the more viscid fraction of egg white. These preparations were found to inhibit trypsin to a much greater degree than leucoprotease (Table V).

TABLE V  
*The Influence of Heavy Metals on the Activity of Leucoprotease and Trypsin*  
(The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture gm./100 cc.	Leucoprotease	Crude trypsin
None (control).....	—	100	100
AlCl <sub>3</sub> .....	2.0	9	20
“ .....	0.05	12	
“ .....	0.02	40	77
“ .....	0.01	103	
Mn(Ac) <sub>2</sub> ·4H <sub>2</sub> O.....	0.20	0	28
“ .....	0.04	0	104
FeSO <sub>4</sub> .....	0.10	0	66
“ .....	0.02	0	97
HgCl <sub>2</sub> .....	0.30	0	0
“ .....	0.04	6	0
ZnCl <sub>2</sub> .....	Suspension 0.05	66	50
CuSO <sub>4</sub> .....	0.05	16	48

VII), a finding which lends support to the belief that although leucoprotease and trypsin are very similar, they are probably not identical enzymes.

An interesting observation was that the boiling of solutions of tryptic digests of proteins increased their power of retarding the activity of trypsin. This is in contrast to serum, which loses its antitryptic and antileucoprotease activity when heated at 80°C. for 10 minutes. These findings will be discussed later, under consideration of the nature of serum antiprotease.

Egg white antitrypsin inhibited crystalline as well as crude trypsin. This disproves the theory of Balls and Swenson (1934) that egg white antitrypsin

acts by inhibiting trypsin kinase, since crystalline trypsin requires no kinase for its action.

TABLE VI

*The Influence of Some Other Substances on the Activity of Leucoprotease and Trypsin*  
(The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture	Leucoprotease	Crude trypsin	Crystalline trypsin
	gm./100 cc.			
None (control).....	—	100	100	100
Serum.....	0.5 per cent	3	3	6
Serum heated at 80° for 10 min.....	0.5 per cent	105	112	108
Plasma.....	0.5 per cent	3	3	7
Plasma heated at 80° for 10 min.....	0.5 per cent	103	110	106
Urea.....	2.0	81	105	
".....	0.5	89	98	
".....	0.2	87	108	
Thiourea.....	1.0	45	112	
".....	0.25	58	106	
".....	0.10	72	94	
Heparin .....	0.06	72	90	
".....	0.015	80	95	
".....	0.006	100	98	
Glutamic acid.....	0.20	20	30	
".....	0.02	54	100	
Quinine mono-HCl.....	0.20	46	19	
".....	0.02	63	91	
Penicillin.....	500 units/100 cc.	78	104	
".....	50 units/100 cc.	73	91	
Tyrothricin.....	0.008	14	70	
".....	0.0008	18	94	
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> .....	0.20	4	70	
".....	0.05	68	84	
".....	0.02	74	104	
".....	0.005	82	95	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	0.05	82	95	
".....	0.01	94	94	
NH <sub>4</sub> NO <sub>3</sub> .....	0.05	72	74	
".....	0.01	89	82	
NH <sub>4</sub> citrate.....	0.10	39	75	
".....	0.02	74	99	
NH <sub>4</sub> oxalate.....	0.10	72	100	
".....	0.02	100	101	
NH <sub>4</sub> tartrate.....	0.10	68	92	
".....	0.02	93	94	

TABLE VI—Concluded

Substance	Concentration in digest mixture	Leucoprotease	Crude trypsin	Crystalline trypsin
gm./100 cc.				
CaCl <sub>2</sub> .....	0.05	53	64	
“ .....	0.02	88	89	
Na <sub>2</sub> SO <sub>4</sub> .....	0.20	68	104	
Asparagine.....	0.40	6	98	
“ .....	0.04	15	104	
Na glycocholate.....	0.10	8	49	
“ “ .....	0.02	21	104	
Na taurocholate.....	0.10	52	98	
“ “ .....	0.02	82	102	
Cholic acid.....	0.10	0	85	
“ “ .....	0.02	11	105	
Desoxycholic acid.....	0.50	6	104	
“ “ .....	0.05	11	108	
Boiled crude trypsin.....	0.004	75	90	
Benzyl HgCl.....	1/50 saturation	66	80	100
p-tolyl HgCl.....	“ “	82	75	90
Hg di-p-Tolyl.....	“ “	84	92	102

TABLE VII

*The Influence of Prepared Trypsin Inhibitors on the Activity of Leucoprotease and Trypsin*  
(The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture	Leucoprotease	Crude trypsin	Crystalline trypsin
gm./100 cc.				
None (control).....	—	100	100	100
Egg white antitrypsin.....	0.2	100	17	35
“ “ “ .....	0.05	101	35	
Boiled egg white antitrypsin.....	0.2	101	17	
Tryptic digest of egg albumen.....	0.2	100	67	84
Boiled tryptic digest of egg albumen.....	0.2	98	37	
Tryptic digest of serum albumin.....	0.2	91	84	86
Boiled tryptic digest of serum albumin .....	0.2	95	73	
Tryptic digest of casein.....	0.2	98	86	35
Boiled tryptic digest of casein.....	0.2	93	47	

#### 4. Substances Which Accelerated Leucoprotease and Trypsin

A number of oxidizing agents were found, in suitable concentration, to increase the activity of leucoprotease and trypsin (Table VIII). This was in direct contrast to their inhibiting effect on papain. Whether the oxidants

exerted their effect directly on the leucoprotease and trypsin, or by oxidizing unknown reductant inhibitors present in the digest mixtures, was not ascertained. Higher concentrations, especially of stronger oxidizing agents, inhibited and could even completely prevent the action of these two proteases.

TABLE VIII

*The Influence of Oxidizing Agents on the Activity of Leucoprotease and Trypsin*

(The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture gm./100 cc.	Leucoprotease	Crude trypsin	Crystalline trypsin
None (control).....	—	100	100	100
L-Cystine.....	0.02	107	120	110
“ .....	0.01	106	112	
“ .....	0.004	103	105	
I <sub>2</sub> in KI.....	0.004 (each)	145	77	83
ICl, tri-.....	0.01	40	20	41
“ .....	0.001	115	106	
IBr.....	0.02	0	0	0
“ .....	0.001	110	105	100
H <sub>2</sub> O <sub>2</sub> .....	3 parts in 100	106	108	103
Dakin's solution.....	1 part in 1,000	107	107	104
Dichloramine T.....	0.015	105	110	105
Azochloramide.....	0.06	121	114	110
NaClO <sub>3</sub> .....	0.40	108	102	
“ .....	0.04	123	115	106
NaBrO <sub>3</sub> .....	0.40	30	85	
“ .....	0.04	105	110	105
Na per ClO <sub>3</sub> .....	0.40	20	105	
“ “ .....	0.04	110	115	107
Cu <sub>2</sub> O (suspension).....	0.40	92	104	
“ “ .....	0.04	104	110	98
Ferricyanide.....	0.20	70	85	
“ .....	0.02	106	104	100
o-Iodosobenzoic acid.....	0.20	82	116	
“ “ .....	0.02	109	118	

The increase of leucoprotease and tryptic activity in the presence of mild oxidizing agents corresponds with the finding of their inhibition by reducing agents, and supports the contention that the oxidation-reduction system is important in the control of the activity of these proteolytic enzymes.

That the oxidation-reduction system may be important in protein synthesis too is suggested by a report (Voegtl<sup>n</sup> *et al.*, 1932) that oxygenation favors the synthesis of protein by papain from protein digests and tissue extracts. Others

(Strain and Linderstrøm-Lang, 1938) have failed to reproduce this finding, but in view of the fact that proteolytic enzymes have been found by Wasteneys and Borsook (1930) to synthesize proteins from split products (by means of equilibrium systems which could continuously remove the synthetic product), it is very possible that the oxidation-reduction system will be found to be significant in protein synthesis, as it is in proteolysis.

### *5. Substances Which Inhibited the Antiproteolytic Activity of the Serum*

The cause of the antiproteolytic activity of the serum has been the subject of much dispute. This activity has been attributed to antibodies to trypsin and/or leucoprotease (Jochman, 1908), to adsorption of the enzyme by serum albumin (Hedin, 1907), to unsaturated fatty acids (Jobling and Petersen, 1914), and to products of protein digestion, probably polypeptide in nature, and probably produced both in the intestine and parenterally (Grob, 1943).

(a) *Inhibitors of the Antiproteolytic Activity of the Serum.*—In Table IX are listed the substances which, in suitable concentration, were found to inhibit the antiproteolytic activity of the serum. These are seen to be, for the most part, oxidizing agents. (Dakin's solution was especially effective, which is interesting in view of its ability to accelerate the solution of pus.) An effort was made to use concentrations of oxidant which decreased rather than increased the activity of the proteases, so as to differentiate between the direct effect on the enzymes and the effect on the antiproteolytic activity of the serum. In addition to the oxidants, three other substances of special interest: zinc chloride, copper sulfate, and *o*-iodosobenzoic acid, inhibited the antiproteolytic activity of the serum. These substances are special inhibitors of sulphhydryl groups (Hellerman *et al.*, 1941).

(b) *The Nature of the Antiproteolytic Substance in the Serum.*—Evidence has been presented that: (1) Leucoprotease and trypsin are readily inhibited by reducing agents, including thiol-sulphhydryl compounds and ascorbic acid; (2) the antiproteolytic activity of the serum is inhibited by oxidizing agents and by some special inhibitors of sulphhydryl groups; and (3) the antiproteolytic activity of the serum is due in part to protein degradation products (Grob, 1943) which are most likely polypeptides.

On the basis of this evidence it is suggested that the antiproteolytic activity of the serum is at least partly due to reducing agents in the serum, including polypeptides (particularly those containing thiol-sulphhydryl compounds such as cysteine) and to a lesser degree ascorbic acid. That the part played by ascorbic acid in this activity is a minor one is indicated by the evidence that the antiproteolytic activity of the serum (as tested against trypsin) differs from ascorbic acid in several respects. The antiproteolytic activity is largely confined to the albumin fraction of the serum (Landsteiner, 1900); it is not dialyzable (Fujimoto, 1918); it is not freely diffusible in the body except when

inflammation or other pathologic process increases capillary permeability (Weinberg and Laroche, 1909); and it could be accounted for to only a small extent by the concentration of ascorbic acid in the serum, which is 1 to 2 mg. per cent when the body is saturated with it. Serum polypeptides, on the other hand, would behave like serum antiprotease if the evidence advanced by Loise-

TABLE IX

*The Influence of Oxidizing Agents and Some Special Sulfhydryl Inhibitors on the Antiproteolytic Activity of the Serum*

(The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture gm./100 cc.	Leucoprotease	Leucoprotease +0.5 per cent serum	Crude trypsin	Cryde trypsin +0.5 per cent serum
None (control).....	—	100	3	100	3
Serum heated at 80° for 10 min....	0.5 per cent	105		112	
Carboxypeptidase (incubated with serum for 24 hrs.).....	Few milligrams	105	23	96	25
ICl, tri.....	0.001	115	20	106	18
" .....	0.0005	98	148	99	30
IBr.....	0.001	110	26	105	8
ICl, mono.....	0.001	101	18	70	6
I <sub>2</sub> in KI.....	0.004 each	145	65	77	9
H <sub>2</sub> O <sub>2</sub> .....	3 parts in 100	106	40	108	14
Dakin's solution.....	3 parts in 1,000	76	67	75	42
Azochloramide.....	0.006	99	16	97	8
Chloramine T.....	0.02	92	2	94	14
Dichloramine T.....	0.015	105	23	110	15
NaClO <sub>3</sub> .....	0.40	108	20	102	6
NaBrO <sub>3</sub> .....	0.40	70	30	85	9
Na per ClO <sub>3</sub> .....	0.40	115	20	105	8
Cu <sub>2</sub> O.....	0.40	92	15	104	6
<i>o</i> -Iodosobenzoic acid.....	0.20	82	4	116	10
" "	0.02	109	4	118	9
ZnCl <sub>2</sub> (suspension).....	0.05	66	18	50	8
CuSO <sub>4</sub> .....	0.05	16	9	48	7

leur and Colliard (1937) that these polypeptides are normally adsorbed to the serum proteins is confirmed, for they would then be fractionated with the proteins; they would not be freely dialyzable; and they would not be freely diffusible in the body except when capillary permeability is increased.

(c) *The Nature of Other Trypsin Inhibitors.*—Reference was previously made to a number of polypeptide trypsin inhibitors which have been prepared from tryptic digests of proteins, and from the thin fraction of egg white. The latter,

egg white antitrypsin, has been studied analytically (Balls and Swenson, 1934) and has been found to be a polypeptide rich in cysteine and in thiol groups. It may well owe its antitryptic activity to the presence of active sulfhydryl groups. The crystalline trypsin inhibitors of pancreas (Northrop and Kunitz, 1932) and serum (Schmitz, 1938) have probably also been studied analytically, but any such data were not readily available.

The increase in the activity of trypsin inhibitor solutions (prepared from protein digests) as a result of boiling is demonstrated in Table VII. It has been shown (Dubos, 1929) that boiling solutions of peptone increases their reducing capacity. This would be expected to increase the antiproteolytic activity, if such activity were due to reducing substances. The destruction of

TABLE X  
*Substances Which Were Found to Have No Effect on the Antiproteolytic Activity of the Serum (Other Than an Additive Effect on the Enzyme Used in Testing)*

Substance	Concentration
Na iodate.....	0.25
Ferricyanide.....	0.20
".....	0.05
<i>L</i> -Cystine.....	0.01
<i>p</i> -Tolyl HgCl.....	1/50 saturation
Benzyl HgCl.....	1/50 saturation
Hg di- <i>p</i> -tolyl.....	1/50 saturation
Sodium selenite.....	0.05
Cysteine.....	0.05
Ascorbic acid.....	0.05
Na oxalate.....	0.05
Na citrate.....	0.05
Sulfathiazole.....	0.012

the antiproteolytic activity of the serum by heat (80°C. for 10 minutes) is not at variance with these findings, since evidence has been presented (Grob, 1943) that this destruction is due to the denaturation by heat of serum proteins (especially albumin), to which serum antiprotease is probably bound.

(d) *Objections to the Reducing Agent-Sulfhydryl Theory of the Antiproteolytic Activity of the Serum, and Further Work That Is Indicated.*—More convincing evidence for attributing the antiproteolytic activity of the serum to the action of sulfhydryl compounds would be the demonstration of inactivation of these compounds by mercaptide-forming reagents (e.g. benzylmercuric chloride, *p*-tolyl mercuric chloride, cuprous oxide), but not by symmetrically substituted mercury compounds (e.g. mercury di-*p*-tolyl), which cannot convert mercaptans to substituted mercaptides. These four reagents were tested, the mercury compounds in concentration of 1/50th saturation, and only the cuprous oxide was found to inhibit serum antiprotease. Table X lists some of the

compounds which, in the concentrations studied, had no observed effect on serum antiprotease. These included three inhibitors of sulfhydryl groups; namely the two mercaptide-forming mercury reagents and sodium selenite. However, a wider range of concentration of these compounds must be tested before it can be said that they are not effective against serum antiprotease.

Serum inhibits papain, though to a much lesser degree than it inhibits trypsin or leucoprotease. The inhibition of papain must be explained by some other mechanism than sulfhydryl groups, since papain is activated rather than inactivated by sulphydryl compounds. It is probable that the antiproteolytic activity of the serum is the sum of two or more factors, and at least one of these factors (ascorbic acid) inhibits papain in spite of the fact that it is a reducing agent that also inhibits trypsin and leucoprotease.

The sulfhydryl theory would be supported by demonstration of the reversibility of the inhibition of serum antiprotease. Experiments were planned: (a) to test the reversibility of the inhibition of serum antiprotease; (b) to test the reversibility of the inactivation of leucoprotease and trypsin by reducing agents and their activation by oxidizing agents; (c) to study the possible inhibition of the trypsin inhibitors prepared from tryptic digests of proteins and from egg white, in a manner similar to the study of the inhibition of serum antiprotease; and (d) to study the antiproteases that might be prepared from leucoprotease digests of proteins. However, time was not available for their execution.

#### *6. The Influence of pH on Leucoprotease, Trypsin, Papain, and Serum Antiprotease*

Leucoprotease has long been known to have its optimum pH at 7, trypsin at 8, and papain at 5 to 6. Northrop (1922) has shown that the inhibition of trypsin by the products of tryptic hydrolysis of proteins is not affected by pH between 6 and 10. Weiss (1927) has demonstrated that adjusting the reaction to a pH of 5 or less causes serum to lose its antitryptic power.

The influence of pH on leucoprotease, trypsin, papain, and serum antiprotease was studied by following the digestion of casein (nephelometrically) in media buffered at various pH values. The results are recorded in Tables XI and XII (and reproduced in Charts 3A, 3B, and 3C). Chart 3A shows the activity of the three proteases at varying pH, and the optima are seen to be pH 8 for trypsin, pH 7 for leucoprotease, and pH 6 for papain, with rather restricted range of activity for leucoprotease and trypsin. Chart 3B shows the activity of the enzymes at various pH values in the presence of serum (0.2 per cent). It is seen that the serum has markedly inhibited trypsin and leucoprotease, and has only slightly inhibited papain. Furthermore the presence of serum has caused the pH optima of leucoprotease and trypsin to shift to the acid side, to a pH of 6 to 6.5, and it has caused the range of activity to widen slightly in both acid and alkaline reactions.

The reason for these latter changes is made clear by study of the effect of exposing serum to different pH values for 1 hour, then adjusting the reaction

TABLE XI

*The Influence of pH on the Activity of Leucoprotease, Trypsin, and Papain, in the Absence of Serum and in the Presence of Serum*

(The per cent of enzyme activity is recorded.)

pH	Leucoprotease	pH	Leucoprotease + 0.2 per cent serum	pH	Crude trypsin	pH	Crude trypsin + 0.02 per cent serum	pH	Papain	pH	Papain + 0.2 per cent serum
1.8	0	2.0	0	2.5	0	2.1	0	2.0	0	3.3	8
3.1	0	3.5	0	4.1	0	4.9	0	4.5	68	4.5	40
4.7	0	5.3	15	5.8	4	5.4	3	5.6	92	5.6	51
6.0	60	5.8	35	6.5	70	5.8	25	6.0	100	6.0	71
6.3	80	6.3	55	6.6	74	6.3	36	6.3	95		
6.5	88			6.8	88	6.8	36	6.8	90	6.7	83
7.0	100	7.4	37	7.4	98	7.4	29	7.4	74	7.4	67
8.0	80	8.0	27	7.9	100	8.0	20	7.9	50	8.0	56
9.2	28	9.2	14	8.6	80			9.2	0	9.2	42
9.5	0	9.8	0	9.1	4	9.1	12	9.5	0		
10.8	0	10.9	0	9.3	0	9.5	0			10.0	0

TABLE XII

*The Influence of pH on the Antiproteolytic Activity of the Serum*

(Serum exposed to indicated pH for 1 hour, neutralized, added to digest mixture, and protease action determined at pH 7.8. The per cent of enzyme activity is recorded.)

pH to which serum was exposed	Leucoprotease + 0.1 per cent serum	Crude trypsin + 0.1 per cent serum	Papain + 0.1 per cent serum
2.6	96	100	100
3.7	96	97	98
4.5	91	90	96
5.9	90	85	94
6.6	85	77	94
7.4	80	75	90
8.0	80	75	90
9.7	85	80	92
10.7	95	88	94
11.2	100	100	95

back to neutral and adding the serum to digest mixtures (to reach a concentration of 0.1 per cent), and determining the protease action (at pH 7.8). The results of this procedure are reproduced in Chart 3C, which shows that the antiproteolytic activity of the serum (against leucoprotease, trypsin, and

papain) is progressively weakened by exposure to hydrogen ion concentrations below pH 6.5 or above pH 9.7.

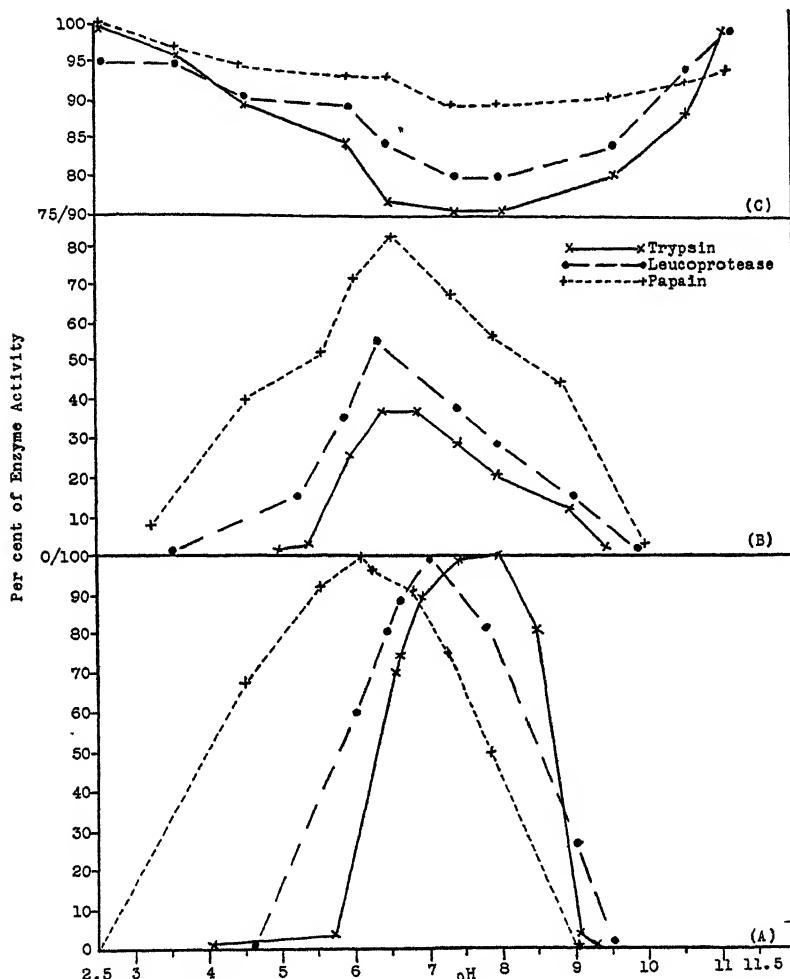


CHART 3. A, the influence of pH on the activity of leucoprotease, crude trypsin, and papain. B, the influence of pH on the activity of leucoprotease, crude trypsin, and papain in the presence of serum (0.2 per cent). C, the influence of pH on the antiproteolytic activity of serum (0.1 per cent). (Serum exposed to indicated pH for 1 hour, neutralized, added to digest mixture, and protease action determined at pH 7.8.)

The activity of protease in the presence of serum and at various pH values will be directly proportional to the activity of the enzyme at any given pH, and inversely proportional to the activity of serum antiprotease at that pH. The resultant activity will be the summation of these two factors. At neutral

pH leucoprotease and trypsin are most active, but serum antiprotease is also at its optimum activity. At a pH of 6 to 6.5 the enzymes are somewhat less active, but the serum antiprotease is weakened to a greater degree, so that the resultant activity in the presence of serum exceeds that at neutral pH. At pH values below 6.5 and above 9.7 serum antiprotease is increasingly inactive, and the presence of serum may then actually help enzyme action by virtue of its buffering power.

The influence of pH on the antiproteolytic activity of the serum, and the influence of the presence of serum on the pH optimum of leucoprotease, are significant because all three factors—leucoprotease, antiprotease, and hydrogen ion concentration—are variables of great importance wherever polymorphonuclear leucocytes accumulate in the body. The interplay of these factors may well explain many of the obscure phenomena of inflammation: for instance, to name only one, why proteolysis frequently proceeds more rapidly in exudates (especially purulent ones) after the accumulation of some protein digest products has rendered the medium *more* acid than the optimal pH for leucoprotease activity.

The hydrogen ion concentration not only exerts a direct influence on the activity of leucoprotease and serum antiprotease, but by shifting oxidation-reduction equilibria exerts an additional and important indirect effect. The sensitivity of oxidation-reduction potentials to hydrogen ion concentration has been fully demonstrated by Clark and his coworkers (1928).

#### D. THE CONTROL OF THE ACTIVITY OF LEUCOPEPTIDASE (EXPERIMENTAL DATA)

Peptidase activity has been found in extracts of human polymorphonuclear leucocytes (Husfeldt, 1931). Human serum has also been found to contain a small amount of active peptidase (Jobling and Strouse, 1912). It is claimed (Jobling, Petersen, and Eggstein, 1915) that serum possesses no antipeptidase activity.

In view of the physiological significance of the enzymes of the polymorphonuclear leucocytes, the control of the activity of the peptidase of these cells ("leucopeptidase") was studied. It was originally intended to repeat with leucopeptidase the experiments described above (performed with leucoprotease). However time permitted only a few preliminary experiments on the control of the activity of leucopeptidase, and of pancreatic peptidase (in crude trypsin).

Leucopeptidase and pancreatic peptidase activity were determined by adding glycerol extracts of cat polymorphonuclear leucocytes (the "leucoprotease" described above), and crude trypsin (Fairchild, beef) to a substrate of peptic digest of edestin (prepared after Anson, 1937), stopping the digestion after a given time by the addition of formaldehyde, and finally adding phenolphthalein and determining how much sodium hydroxide was needed to make the solution as pink as a standard solution. The titration was begun with

strong alkali and completed with 0.02 N NaOH, in terms of which the results are recorded. This was done in order to avoid increasing the volume, which would produce the so called "water error" (Harris, 1923).

TABLE XIII

*Preliminary Experiment on the Influence of Reducing and Other Substances on Leucopeptidase and Pancreatic Peptidase*

(Enzyme activity is recorded as the cubic centimeters of 0.02N NaOH (or its equivalent) required to titrate digest mixture of 8 cc. peptic digest of edestin + 1 cc. enzyme + 1 cc. substance to be tested, after 20 hours incubation at 37°C. under toluene.)

Substance	Concentration in digest mixture gm./100 cc.		
		Leucopeptidase	Pancreatic peptidase
None (control).....	—	3.0	4.0
Cysteine.....	0.03	5.2	5.6
Ascorbic acid.....	0.05	6.3	7.4
Serum.....	1.0 (per cent)	4.0	4.8
Sulfathiazole.....	0.010	3.1	3.9
Thiourea.....	0.50	2.5	2.8

TABLE XIV

*Summary of the Influence of Reducing and Oxidizing Agents on the Systems Studied*

Source	Enzyme	Influence of reducing agents	Influence of oxidizing agents
Polymorph. leucocytes . . . . .	Leucoprotease	—	+
	Leucopeptidase	+	(Not studied)
Pancreas . . . . .	Trypsin (proteinase)	—	+
	Erepsin (peptidase)	+	(Not studied)
Papaya plant . . . . .	Papain proteinase	+	—
	" peptidase	— (Bergmann and collaborators, 1935-36)	+(Bergmann and collaborators, 1935-36)
Serum . . . . .	Enzyme inhibitor		
	Antiprotease	+ (Additive)	—

+= increased activity.

- = decreased activity.

The influence of cysteine, ascorbic acid, serum (human), sulfathiazole, and thiourea on leucopeptidase and pancreatic peptidase was studied. Results are recorded in Table XIII. The few experiments that were performed indicate that extracts of cat polymorphonuclear leucocytes contain active peptidase, and that the activity of this leucopeptidase, as well as of pancreatic

peptidase, is increased by reducing agents such as cysteine and ascorbic acid, and is not influenced appreciably by serum, sulfathiazole, or thiourea. (The slight increase in activity caused by serum may be due to serum peptidase.)

The presence in extracts of polymorphonuclear leucocytes and of pancreas of proteinase (leucoprotease and trypsin) which is inhibited by reducing agents, and of peptidase (leucopeptidase and erepsin) which is activated by reducing agents, recalls another reciprocal relationship in the control of the activity of proteinase and peptidase: namely, the presence in crude papain preparations of proteinase which is activated by reducing agents and peptidase (Bergmann and collaborators, 1935-36) which is inactivated by reducing agents. Table XIV lists these enzymes (and serum antiprotease) and summarizes the influence of reducing and oxidizing agents. The reciprocal relationships that exist in the control of these enzyme systems are no doubt significant physiologically, and perhaps teleologically as well.

#### SUMMARY

1. The literature on conditions affecting the activity of proteolytic enzymes has been reviewed.

2. Experimental data on the control of the activity of trypsin, leucoprotease, papain, serum antiprotease, leucopeptidase, and pancreatic peptidase have been presented. These data indicate that:

(a) The polymorphonuclear leucocytes of the cat contain abundant proteinase and peptidase active at neutral pH; those of the rabbit lack proteinase active at neutral pH.

(b) Reducing agents, including several biologically important thiol-sulfhydryl compounds and ascorbic acid, inhibit the activity of leucoprotease and trypsin. For each reductant the degree of inhibition is proportional to the reducing capacity of the medium.

(c) *p*-Aminobenzoic acid, sulfonamides (especially sulfathiazole), and many diphenyl sulfones inhibit the activity of leucoprotease.

(d) Serum, plasma, several heavy metals, ammonium salts, asparagine, thiourea, heparin, glutamic acid, tyrothricin, calcium chloride, and bile salts and bile acids also inhibit the activity of leucoprotease, and in most cases of trypsin too.

(e) Preparations of tryptic digests of proteins, and egg white trypsin inhibitor, inhibit trypsin to a much greater degree than leucoprotease.

(f) Mild oxidizing agents increase the activity of leucoprotease and trypsin.

(g) Oxidizing agents and some inhibitors of sulfhydryl groups inhibit the antiproteolytic activity of the serum. It is suggested that serum antiprotease may consist (chiefly) of reducing agents, including thiol-sulfhydryl peptides which exert their antiproteolytic activity by virtue of the presence of sulfhydryl groups.

(h) The antiproteolytic activity of the serum is progressively weakened by exposure to a hydrogen ion concentration below pH 6.5 or above pH 9.7. Because of this the pH optima of leucoprotease and trypsin are shifted in the presence of serum from pH of 7 and 8 to pH of 6 to 6.5, and the range of activity of these enzymes is slightly widened, in both acid and alkaline reactions.

(i) Reducing agents increase the activity of leucopeptidase and pancreatic peptidase. Serum, sulfathiazole, and thiourea have little or no effect.

3. The significance of the oxidation-reduction system in the control of the activity of leucoprotease, trypsin, serum antiprotease, leucopeptidase, and pancreatic peptidase has been emphasized.

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## PROTEOLYTIC ENZYMES

### II. THE PHYSIOLOGICAL SIGNIFICANCE OF THE CONTROL OF THEIR ACTIVITY, ESPECIALLY WITH RESPECT TO BACTERIAL GROWTH

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Müller (1888) first described the presence in human purulent sputum of protease active at neutral pH. Achalme (1899) was the first to find this enzyme in human pus. Jobling and Strouse (1912) showed that extracts of human pus also contain peptidase active at neutral pH. Opie (1905, 1922) showed that polymorphonuclear leucocytes are the chief source of leucoprotease (named by him) and was the first to emphasize the physiological significance of this protease and of its inhibition by serum. He demonstrated that leucoprotease plays a major rôle in the resolution of inflammatory exudates, and that serum (in sufficient concentration) can retard this resolution by virtue of its inhibitory power. More recent workers (Holmes *et al.*, 1935) have applied these principles to the study of suppurative arthritis and have shown that the presence of antiproteolytic substances in synovial exudates is of great importance in the prevention of damage to joints by protease liberated from the polymorphonuclear leucocytes of these exudates. However, the general problem of the relation of leucoprotease and serum antiprotease to the resolution and absorption of inflammatory exudates and absorbable foreign bodies has not been intensively studied since the work of Opie early in the century. It is hoped that the experiments described in the preceding paper on the control of leucoprotease and serum antiprotease will facilitate such a study by enabling better control of the activity of these important substances than has hitherto been possible.

The experiments of Menkin (1938) and others indicate that the increased capillary permeability and leucocyte infiltration characteristic of inflammation are due to a polypeptide formed by proteolysis at the site of inflammation. Evidence has also been presented (Rocha e Silva, 1941, 1942) that local proteolysis in the tissues liberates histamine, which is thought by some to be important in inflammation and anaphylaxis. The ability to control the activity of leucoprotease and of serum antiprotease may perhaps facilitate further investigation of these phenomena.

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Other physiological and pathological processes in which the ability to control the activity of leucoprotease (or other proteases) and serum antiprotease may prove to be of value include:

(1) The neutralization of the destructive protease liberated in acute pancreatitis and from duodenal fistulae.

(2) The experimental production (Rich and Duff, 1937) of hyaline arteriosclerosis and arteriolonecrosis by subcutaneous injection of proteolytic enzymes. (The local necrosis produced by trypsin has been shown to be inversely proportional to the antiproteolytic activity of the serum (Grob, 1943).)

(3) The coagulation of the blood. Eagle and Harris (1936-37) have presented evidence that, in the course of physiological coagulation, calcium plus thromboplastin constitutes a proteolytic enzyme system (analogous to trypsin) which is believed to be responsible for the transformation of prothrombin to thrombin, itself thought to be a proteolytic enzyme that accelerates the transformation of fibrinogen to fibrin. More recently evidence has been presented (Grob, 1943) that purified serum antitrypsin and pancreatic trypsin inhibitor inhibit the coagulation of plasma *in vitro*.

(4) The possibility of prolonging insulin action by retarding the breakdown of insulin by serum protease (and other proteases?).

(5) Possibly the release of thyroglobulin by the thyroid gland. Para-aminobenzoic acid, sulfonamides, and thiourea, which have been shown to inhibit leucoprotease, are known (Mackenzie and Mackenzie, 1943; Astwood *et al.*, 1943) to inhibit the activity of the thyroid gland. Dziemian (1943) was able to correlate the activity of the thyroid gland of rats with the proteolytic (catheptic) activity of thyroid extracts. He found that sulfaguanidine decreased both activities, and presented evidence in support of the suggestion of Gersh and Caspersson (1940) that a proteolytic enzyme in the thyroid hydrolyzes thyroglobulin into peptones or polypeptides which are small enough to pass across the cell membranes and are then reconstituted by enzyme action into a larger molecule.

(6) Bacterial growth and sulfonamide action (discussed below).

In a previous paper (Grob, 1943) evidence was presented that:

(1) Heating diluted serum at 80°C. for 10 minutes makes it a better medium for bacterial growth. This is believed to have been at least partly due to destruction of the serum antiprotease by heat. (2) Growth is accelerated and proceeds further in the presence of trypsin. (3) Growth is somewhat retarded in the presence of pancreatic trypsin inhibitor. (4) The bacteriostatic action of sulfathiazole in serum is reduced by heating the serum at 80°C. for 10 minutes and much more markedly by adding trypsin. It is greater in serum and albumin than in peptone and meat infusion.

These findings were taken to indicate that the products of tryptic digestion promote bacterial growth, and directly and indirectly inhibit the bacteriostatic action of sulfathiazole. On the basis of these findings it was concluded that the growth of bacteria

in the body will be more rapid, and the inhibition of sulfathiazole will be greater: (a) when considerable leucoprotease (and peptidase?) is present; (b) when the organism itself produces active protease (and peptidase?); (c) when the inflammatory exudate is small and poor in antiprotease; (d) when the source of antiprotease (the blood) is poor in this constituent; and (e) when the medium is rich in non-protein nitrogen (especially para-aminobenzoic acid?).

These conclusions would not be valid if leucoprotease could not be shown to have the same effect on bacterial growth and sulfonamide action as was shown for crude trypsin. To investigate this the growth of *Escherichia coli*, *Proteus vulgaris*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Clostridium welchii* was followed in the following media:

- (1) 10 per cent human serum (serum diluted with isotonic phosphate buffer and heated at 56°C. for 30 minutes to destroy complement, so as to eliminate the action of normal bactericidal antibodies).
- (2) 1 heated at 80°C. for 10 minutes to destroy antiproteolytic activity.
- (3) 1 + sulfathiazole (0.1 mg./cc.).
- (4) 2 + sulfathiazole (0.1 mg./cc.).

Growth was followed in these media in the absence of added enzyme, and in the presence of crude trypsin (1.0 mg./cc.), crystalline trypsin (0.25 mg./cc.), and leucoprotease (cat, lyo-, equivalent to 0.2 mg./cc. crude trypsin). Bacterial growth was determined by measuring the turbidity of bacterial suspensions after 24 hours' incubation at 37°C. by means of a photoelectric nephelometer. The opacity of the suspension is represented as the number of 24 hour old organisms (determined by direct count) which, when suspended in the same medium, allowed the same amount of light to be transmitted. A small correction was made for changes in the opacity of the medium resulting from incubation alone (especially for heated serum plus enzyme), by incubating under toluene control tubes of similar composition. The error produced by the digestion of dead bacteria by protease (resulting in some decrease in turbidity) was not corrected for, but several pour plate counts of viable bacteria indicated that this error did not affect the conclusions drawn from the experiment.

The initial concentration of bacteria in each case was approximately 5 million/cc. Except for *Clostridium welchii* (which was obtained from thioglycollate medium) the organisms were obtained from 48 hour old agar slant cultures, and all were washed repeatedly in saline before addition to the test media. *Clostridium welchii* was grown under vaseline to insure anaerobic conditions.

Examination of the data (Table I) reveals that:

- (a) Growth proceeded further in serum that had been heated at 80°C. for 10 minutes to destroy serum antiprotease than in serum that had merely been heated sufficiently to destroy complement. (However, the destruction of serum antiprotease cannot be said to be alone responsible for the increased growth, since denaturation of proteins by heat alters the rate at which they are hydrolyzed, as well as many other of their properties.)

(b) Growth proceeded further in media to which crude trypsin, crystalline trypsin, or leucoprotease had been added.

(c) The bacteriostatic action of sulfathiazole was: (1) somewhat reduced by destruction of the antiproteolytic activity of the serum (by heat, which altered the serum in other ways too); (2) reduced by the presence of crude trypsin, leucoprotease, or (to a lesser extent) crystalline trypsin; (3) not

TABLE I  
*The Influence of Proteolytic Enzymes and Serum Antiprotease on Bacterial Growth and Sulfonamide Action*

Organism.....		<i>Escherichia coli</i> , millions/cc. after 24 hrs.		<i>Proteus vulgaris</i> , millions/cc. after 24 hrs.		<i>Staphylococcus aureus</i> , millions/cc. after 24 hrs.		<i>Streptococcus pyogenes</i> , millions/cc. after 24 hrs.		<i>Clostridium welchii</i> , millions/cc. after 24 hrs.	
Antiproteolytic activity of the serum.....		Intact	0	Intact	0	Intact	0	Intact	0	Intact	0
Enzyme	Sulfathiazole										
None	0	115	380	125	155	105	260	90	145	180	280
	0.1 mg./cc.	70	125	25	55	45	145	45	70	80	85
Crude trypsin	0	165	580	135	185	125	385	165	165	305	320
	0.1 mg./cc.	175	264	25	165	85	165	65	75	300	265
Crystalline trypsin	0	125	520	145	285	105	265	180	325	265	270
	0.1 mg./cc.	125	185	45	40	65	140	40	70	165	200
Leucoprotease	0	260	580	145	300	105	445	180	350	225	245
	0.1 mg./cc.	95	185	75	200	65	345	85	70	185	225
Boiled leucoprotease	0.1 mg./cc.		125		55		150		90		100

influenced by boiled leucoprotease, which also had no appreciable effect on bacterial growth.

These results indicate that the products of protein hydrolysis by leucoprotease accelerate bacterial growth and inhibit sulfonamide action, in a manner similar to that previously described for the products of protein hydrolysis by crude trypsin. They also suggest that while the products of hydrolysis by crystalline trypsin accelerate bacterial growth to about the same extent as those of crude trypsin, the former (higher) degradation products are not as effective in the inhibition of sulfonamide action.

Evidence was previously presented (Grob, 1943) that trypsin inhibitor possesses no bactericidal activity, and trypsin no antibactericidal activity. Although corresponding experiments were not performed for leucoprotease, it is probable that this protease would also be found to have no bactericidal or

antibacterial activity. However, the polymorphonuclear leucocytes of many animals do contain a bactericidal substance ("leukin"), which may be identical with lysozyme (Barnes, 1940) and which will be present at the site of inflammation and in extracts of the polymorphs.

The inhibitory effect of peptone and of various bacterial extracts on sulfonamides has been the subject of intensive study (Lockwood, 1938; McIntosh and Whitby, 1939; Stamp, 1939). Woods (1940) has advanced evidence that this inhibitory effect is due to the presence of para-aminobenzoic acid, and has attributed to the presence of para-aminobenzoic acid preformed in the medium the observed (Lockwood *et al.*, 1937) difficulty of inhibiting bacteria by the use of sulfonamide in peptone and in lesions characterized by considerable tissue destruction. MacLeod (1940) has correlated sulfonamide inhibition with the degree of tissue autolysis (which, he believes, liberates para-aminobenzoic acid from a preexisting conjugated form). Others (Lockwood and Lynch, 1940) have pointed out that organisms known to be susceptible to sulfonamides are in general not actively proteolytic, and it is believed (MacLeod, 1940) that they are probably not capable of producing much para-aminobenzoic acid.

Evidence has been presented that para-aminobenzoic acid also inhibits the "bacteriostatic" action of sulfonamides (Fitzgerald and Feinstone, 1943) and promin (Steenken and Heise, 1943) on tubercle bacilli, and of sulfonamides on the *Plasmodium* of bird malaria and on the virus of lymphogranuloma venereum (Maier and Riley, 1942).

Green and Bielschowsky (1942) have emphasized that the antisulfonamide activity of bacterial and yeast extracts is exerted directly by para-aminobenzoic acid and similar sulfonamide inhibitors, and indirectly by substances which stimulate bacterial growth, even in an optimal nutrient medium. (Para-aminobenzoic acid itself stimulates the growth of very few organisms (Rubbo and Gillespie, 1940; Sevag and Shelburne, 1942; Rantz, 1942).) These latter substances which stimulate growth will also exert their indirect inhibiting effect against other bacteriostatic agents than the sulfonamides; *e.g.*, against penicillin (Green and Bielschowsky, 1942).

Not only the properties of the medium, but also the properties of the bacteria influence sulfonamide action. It is believed (Woods, 1940; Green and Bielschowsky, 1942) that the degree of sensitivity of bacteria to sulfonamides is determined by the extent to which para-aminobenzoic acid and related "essential metabolites" are necessary to the life cycle of the bacteria, and by the rate at which para-aminobenzoic acid is synthesized and released into the surrounding medium. Many investigators have attempted to discover a chemotherapeutic agent which would react with para-aminobenzoic acid, and/or reduce the rate of synthesis of para-aminobenzoic acid by bacteria. It is thought that azochloramide and related halogen compounds (Schmelkes and Wyss, 1942), and perhaps urea (Tenenberg *et al.*, 1942) are capable of neutralizing the action of para-aminobenzoic acid and may thereby prove to be useful as potentiators of the sulfonamides.

The experiments on leucoprotease described above indicate that leucoprotease activity, such as occurs at any site of bacterial growth with its concomitant inflammation, results in the production of protein degradation products which

stimulate bacterial growth, and directly and indirectly inhibit sulfonamide action. (A third way in which leucoprotease activity increases bacterial growth in the presence of sulfonamides is by causing an accumulation of acid products of hydrolysis, which decrease sulfonamide action since sulfonamides have been shown (Schmelkes *et al.*, 1942) to be less active at decreased pH. Of course, when the pH falls too low, as in a poorly buffered medium, bacterial growth will be retarded.)

It is to be expected that substances which inhibit the activity of leucoprotease (*e.g.*, serum, reducing agents, heavy metals, etc.) will inhibit the production of protein degradation products at the site of bacterial growth and inflammation, and will thereby act to prevent the acceleration of bacterial growth, and the inhibition of sulfonamide action, that these products cause. It is suggested that the search for sulfonamide potentiators be directed not only toward the discovery of agents which will destroy sulfonamide inhibitors once formed, but also toward the utilization of substances which will prevent the formation of these inhibitors (and of the degradation products which stimulate bacterial growth) by retarding the action of leucoprotease (and perhaps of bacterial proteases as well). This would seem to be especially desirable in purulent lesions, where the sulfonamides have proved least efficacious. With respect to the use of bacteriostatic agents which are not inhibited in purulent lesions (*e.g.* penicillin) it would still seem desirable to control the production by proteolysis of degradation products which have been shown to directly stimulate bacterial growth. It is hoped that the data presented in the preceding paper on the control of the activity of leucoprotease and serum antiprotease may prove applicable in these respects.

#### SUMMARY

It has been suggested that the ability to control the activity of leucoprotease and serum antiprotease may prove useful in the further study and understanding of such phenomena as: (1) inflammation; (2) the resolution of inflammatory exudates and the absorption of absorbable foreign bodies; (3) the protection of joint and other structures from the proteolytic action of leucoprotease; (4) the neutralization of the destructive protease liberated in acute pancreatitis and from duodenal fistulae; (5) experimental arteriosclerosis and arteriolonecrosis; (6) the coagulation of the blood; (7) the possibility of prolonging the action of insulin; (8) the release of thyroglobulin by the thyroid gland; (9) bacterial growth and sulfonamide action.

In this last respect evidence has been presented that the products of the hydrolysis of protein by leucoprotease stimulate bacterial growth and directly and indirectly inhibit sulfonamide action, and the hope has been expressed that the ability to control leucoprotease action may contribute to the more successful use of chemotherapeutic agents in purulent lesions.

It is a pleasure to acknowledge again my gratitude to Dr. J. Howard Brown for his encouragement and assistance in the performance of these and previous experiments. I am also indebted to Dr. Leslie Hellerman for the interest he has taken in this work, and for the help he has given.

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## NERVE AS MODEL TEMPERATURE END ORGAN

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Transmission across the artificial synapse formed by the cut end of a mammalian nerve is greatly enhanced by cooling the nerve end locally (Granit and Skoglund, 1945). Considering that cooling has increased the electronegativity of this region relative to a region at normal temperature (Grützner, 1881; Verzar, 1911) it seemed worth while to investigate if by these means local cooling of a nerve could set up a discharge. It is well known that cold and heat can stimulate a nerve, and many of those who have been recording impulses from mammalian nerve twigs must have noticed effects on the discharge of dropping too cold or too hot Ringer solution on the preparation. But we are not aware of any recent systematic analyses of such effects nor have they ever been related to temperature potentials.

Bernhard, Granit, and Skoglund (1942) have put forth the general theory that sense organs stimulate their nerves by means of the electrotonic potential conducted from the end organ down the fibres depolarizing the latter. In addition, nerve serving as model sense organ has been studied in several contributions from this laboratory in which instantaneously and slowly rising electrical stimuli have been used (Skoglund, 1942; Granit and Skoglund, 1943; Skoglund, 1945). It is now attempted to find out whether or not stimulation of a nerve by temperature variations can be fitted into this model.

### *Technique*

The main stem of the sciatic nerve of decerebrate or chloralosed cats was locally cooled or warmed by a thermode and the discharge recorded from one of the roots, generally from the sensory L7. The thermode was a small, lacquered metal container with a cross-section like a shallow U forming a groove for the nerve and closed from above by a thin metal lid. The length of the canal for the nerve was 2 cm. The temperature of the thermode was regulated with running water and measured by a thermocouple placed at the bottom of the groove for the nerve and connected to a galvanometer. By pressing a button the experimenter marked on the film the moment when, during warming or cooling, the galvanometer spot passed a scale unit. This meant that every fourth degree of change was recorded.

The condenser-coupled amplifier for the massed discharge from the root was led to a loudspeaker as well as to a diode rectifier for integrating the total effect. This way of measuring irregular spike activity in a large number of fibres seems more satisfactory than trying to count the individual spikes. To begin with, the experiments were

restricted to correlating the temperature at the thermode with the total integrated discharge but in the fully developed experiment the "temperature potential" was measured too. Silver-silver chloride electrodes were taken to the midpoint of the cooled or warmed region (the lid of the thermode having been removed) and to a point on the nerve about 5 cm. farther up. These were connected to a condenser-coupled amplifier capable of recording fairly well the initial phase of the slow temperature potential. The amplifier response to a calibration potential fell to half its full value in 10 seconds. Some controls were made with an electrometer, consisting of two push-pull coupled electrometer valves operating a moving coil galvanometer. With this apparatus it became possible to see how well the temperature potential was maintained. In the standard experiments, however, the temperature potential was recorded by the condenser-coupled amplifier operating one beam of a double-ray cathode ray oscilloscope whereas the other beam recorded the integrated massed discharge from the root.

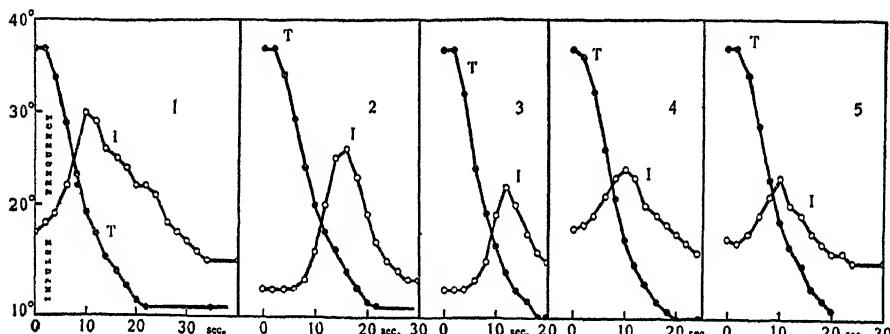


FIG. 1. Five successive observations (1-5) on the effect of local cooling of cat's sciatic upon discharge, I, recorded from root, as described in text. Ordinates: temperature of thermode (Curve T) and integrated relative frequency of nerve impulses (Curve I). Abscissae: time in seconds.

The fully developed technique utilizing the double-ray oscilloscope as described above, became necessary in order to correlate the temperature potential with the impulse discharge. But several simpler problems were taken up before this stage was reached and for these cathode ray and photographic recording were not employed. One experimenter read off the temperature on the galvanometer connected to the thermocouple, the other one the values on a galvanometer connected to the diode rectifier integrating the total impulse frequency. A metronome was used for synchronizing the readings and marking time.

#### RESULTS

*The General Effect of Local Cooling and Warming.*—The experiment shown in Fig. 1 was carried out with a decerebrate cat. The thermode was on the intact sciatic at the knee, the hamstring nerve severed, and the motor and sensory roots L7 cut and isolated.

Chart 1 of Fig. 1 illustrates the stimulating effect of a rapid fall of tempera-

ture. The discharge has been recorded from the sensory L7. In Chart 2 the recording electrodes have been shifted to the motor root. There is less spontaneous activity on the motor side—which, of course, is typical—as shown by the lower initial level of the impulse integrator. The excitatory effect of local cooling passes over in a shorter time. In Chart 3 the sciatic stem has been tied  $1\frac{1}{2}$  cm. from the lower edge of the thermode and the experiment repeated. In Chart 4 the same experiment has been carried out with the electrodes shifted to the sensory L7. Finally, in the experiment of Chart 5, the nerve has been cut at the lower edge of the thermode. This led to a great increase of spontaneous activity lasting for a few minutes. When this effect had disappeared the experiment of Chart 5 was carried out in the same manner as before. The gradual diminution of the effect of cooling from 1 to 5 is typical and need not be ascribed to the severance of the nerve. The artificial end organ always works best in the beginning of an experiment. Repetition diminishes the discharge. The five experiments were carried out in succession and the nerve at the thermode warmed up between each of them.

It is clear that local cooling of a nerve is an effective stimulus capable of causing a massive but transitory discharge, provided that the nerve is in good condition and the animal not too deeply anesthetized. Generally about 2 hours were allowed for disappearance of the immediate effects of the initial anesthesia. When chloralose was given the dose was kept below 6 cc./kg. of a 1 per cent solution.

It is seen in Fig. 1 that the excitatory effect of local cooling is given both by intact nerve and cut end. When the thermode was at the cut end the spontaneous activity from this region was a good index of the general state of the autorhythmic mechanism responsible for the discharge itself. For this reason severed nerves were later used in nearly all experiments. All experiments agreed in showing that the final effect of cooling the nerve locally to some 8–12° C. consisted in practically complete cessation of impulse generation within the cooled region.

It is therefore concluded that the excitation caused by local cooling, is counteracted by an opposite process of immobilization of the discharging mechanism of a nerve. The completeness of this immobilization is not very well shown by Fig. 1 because the cut hamstring nerve continues to discharge so that the integrator never falls down to zero. But it is well brought out by Fig. 5 showing the integrator connected to the cathode ray oscillograph and was regularly confirmed by looking at the massed discharge on the screen of the cathode ray and by listening to it in the loudspeaker. The baseline, broadened by spontaneous and induced activity from the cut end, shrank to a thin line after the transient excitatory effect of cooling and remained so as long as the thermode was kept cold.

If the cooled nerve was warmed through the thermode at the same rate at which it had been cooled and thus brought back to its original temperature

between 36 and 40°, the spontaneous firing from the cut end was resumed. But this effect was late in starting. A latency of some 30 to 120 seconds preceded recovery of the autorhythmic mechanism to be compared with the latent period of a few seconds, that preceded the excitatory effect of cooling.

There is thus, as one would expect, a general effect of temperature on the autorhythmic mechanism of discharge in the sense that cold suppresses and warmth enhances the spontaneous activity set up by the cut end. This general

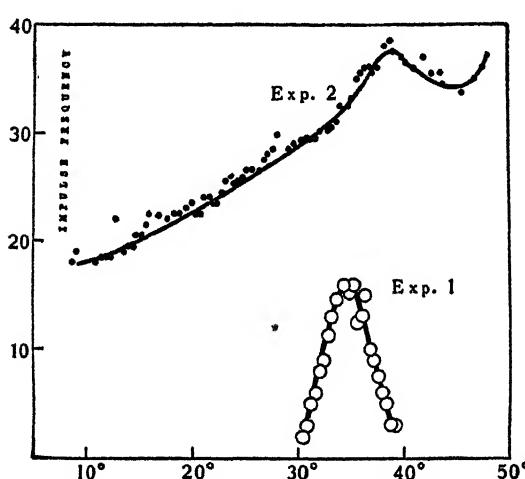


FIG. 2

FIG. 2. Integrated frequency of discharge (ordinates) from cut end of sciatic during slow warming of thermode (abscissae in degrees Centigrade) at the nerve end. Two different animals.

FIG. 3. Depression by warming. Ordinates: temperature (Curve T) in degrees Centigrade and integrated relative frequency of nerve impulses (Curve I). Abscissae: time in seconds.

effect can be isolated and studied alone if the temperature changes are brought on very slowly.

It could easily be shown that stimulation by cold was a function of the rate of change of temperature because if the thermode was cooled very slowly the excitatory effect was negligible, and the impulse activity gradually subsided. Such experiments were used in order to find out whether an optimum temperature could be demonstrated. Fig. 2 shows two experiments in which there were optima in the sense that a slow *increase* of temperature, beyond 40° in the one case, and beyond 36° in the other, again led to a diminution of the spontaneous discharge. The former experiment also demonstrates that a further increase

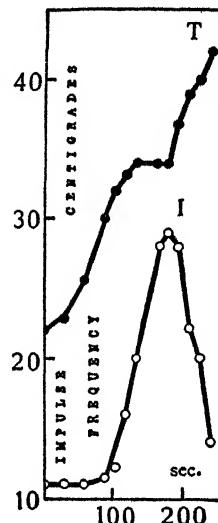


FIG. 3

of temperature above normal re-activated the nerve. This was, in fact, the most regular effect of warming the nerve beyond the maximum which corresponds to the upper limit of body temperature. This re-excitation at high temperatures, which for the moment we shall neglect, was not, as a rule, noticeable below 44° but may, of course, already below that temperature have contributed to the blotting out of the optimum.

The main point to be raised here is that an optimum, when present, shows that local warming of the cut end not only excites but also may inhibit the spontaneous discharge though the inhibitory effect is difficult to demonstrate because it is always pitted against the general favourable effect of raising the temperature. Fig. 3, however, shows one of the cases in which the spontaneous discharge was suppressed by a moderate fast rise of temperature.

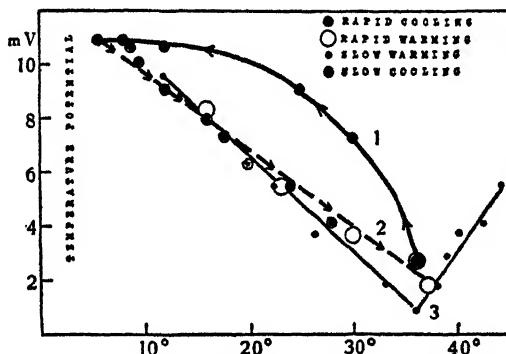


FIG. 4. Temperature potentials, recorded as explained in text.

The general conclusions reached in this section are thus that, apart from the expected favourable effect of warmth and unfavourable effect of cold on the discharge from the cut end, cold causes a strong transitory excitation and warmth a less definite but sometimes demonstrable inhibition. All these observations refer to temperatures at or below the normal range.

*The Temperature Potentials.*—It is known that a cooled region of a nerve becomes negative relative to some point at normal temperature (Grützner, 1881; Verzar, 1911). Since no D.C. amplifier was available for use with the oscillograph, two electrometer valves were set up in push-pull coupling in order to find out how large these potentials were and how well they were maintained in our experiments. The temperature potentials were recorded by a sensitive moving coil galvanometer on a scale on which 1 mm. corresponded to 0.91 mv. Only two animals were used with this technique. Most experiments on temperature potentials were carried out with the condenser-coupled amplifier connected to the oscillograph (see Technique).

The result of the experiment giving the larger temperature responses is shown in Fig. 4. The points, marked 1, are the first set of observations. The ther-

mode temperature was originally  $36^{\circ}$ . In 15 seconds the temperature was then brought down to  $8^{\circ}$  and this made the nerve end more negative by 8.2 mv. Further cooling to  $5.6^{\circ}$  did not increase the negativity which was maintained for 1 minute. The temperature at the thermode was next (Curve 2) increased to  $36^{\circ}$  in 15 seconds. The potential returned to its original value along the curve marked 2. This final value was also maintained. Then followed slow cooling during 9 minutes, the points falling along Curve 3 together with those marking the effect of the final slow warming of the nerve during much the same length of time. The temperature was ultimately increased to  $44^{\circ}$  and it is seen that in this region of supernormal temperature the nerve again responded by increased negativity of the region in the thermode relative to normal nerve.

Thus, from low temperature to the normal range the nerve works like a kind of thermocouple the "cold junction" becoming electronegative relative to the "warm junction." Above the normal range its properties are reversed.

Seeing that the nerve discharges not only when cooled but also when heated above the normal range and that in both these cases the discharging region becomes electronegative relative to a portion at normal temperature it was held to be of great interest to record the temperature potential and the discharge simultaneously, and with sufficiently fast instruments, in order to ascertain whether the electronegativity precedes the discharge or not. This was carried out by means of a second condenser-coupled amplifier, taken to the second beam of the double cathode ray oscillosograph, as described in the section on Technique.

In the record of Fig. 5 the cold potential and the integrated impulses discharged by the cooled end are photographed together. This shows that the cold potential began to rise *before* the rise of the discharge set in. The excitatory effect of cold on the discharge was found to follow between, on an average, 2.2 to 3.4 seconds after the first sign of the rise of a cold potential. The shortest interval noted was 1.4 seconds but this was in an experiment with extremely high amplification for the integrator so that the baseline was rather irregular owing to the spontaneous activity from the cut end. This is also seen to cause oscillations of baseline before cooling, in the experiment of Fig. 5. The unusually short latency of 1.4 seconds in one experiment was probably due to a spontaneous volley just preceding the effect of cold.

The simultaneous records thus showed that without exception the transitory discharge, elicited by local cooling of the nerve began when a certain amount of potential difference had been set up between the cooled point and normal nerve, just as the optic nerve begins to discharge in response to a stationary potential across the sense cells. The temperature potentials recorded by this technique were smaller than those obtained in the electrometric experiments. When corrected for the exponential character of the amplifier response (see Kohlrausch, 1930) the temperature responses were around 0.1 to 0.15 mv. per

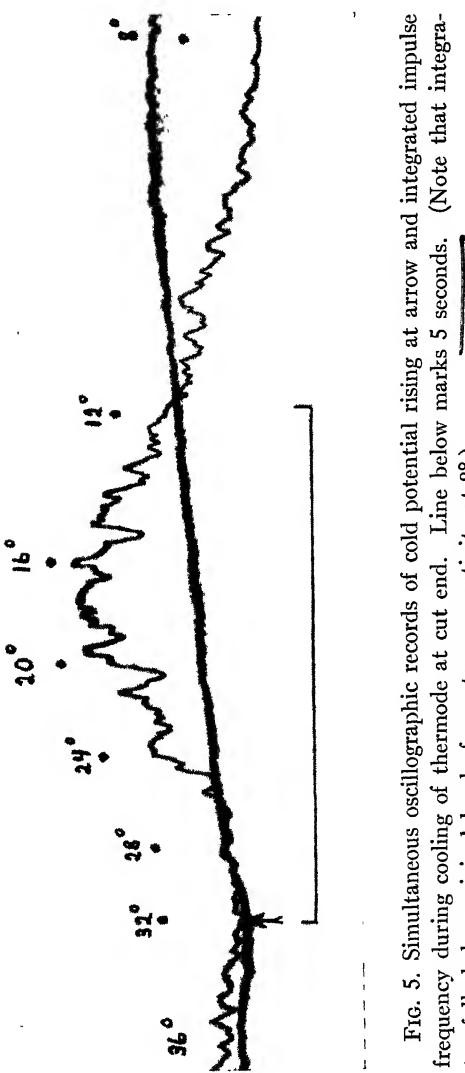


FIG. 5. Simultaneous oscillographic records of cold potential rising at arrow and integrated impulse frequency during cooling of thermode at cut end. Line below marks 5 seconds. (Note that integrator falls below original level of spontaneous activity at 8°.)

degree as against up to 0.3 mv. per degree with the electrometer valves. Generally the potential difference was maintained as long as the low temperature was maintained.

On repetition of such experiments several times in succession it was found that the autorhythmic mechanism of the nerve gradually became less sensitive to stimulation by cold. The temperature potential also diminished somewhat but it often happened that the nerve almost had lost its capacity to respond to cooling by impulse activity at a time when the cold potentials still were almost normal.

#### DISCUSSION

When speaking of nerve as model temperature end organ this is not done in order to call attention to the general unfavourable effect of cold on the spontaneous activity and to the restoration of this activity by warming up the nerve to normal temperature. This finding is merely regarded as an expression of general temperature effects of which no more need be said in this connection. The nerve serves as a model cold end organ in the sense that rapid local cooling sets up a discharge of impulses which is preceded by a potential change of such a character that a discharge must be expected from the cooled region. It is assumed that this potential difference, making the cooled end negative to the rest of the nerve, generates the discharge and that the maintained state of electronegativity would continue to do so, merely hampered by accommodation, were it not for the fact that cold has a general unfavourable effect on the autorhythmic mechanism. Hence the transitory character of the discharge.

Warming the "artificial end organ" removes this generator potential. The circumstance that warmth has a general favourable effect on the discharge counteracts the diminution of the impulse activity that otherwise would be a consequence of diminished electronegativity of the "end organ." Under favourable conditions, however, this opposite effect of warmth relative to cold can be distinguished. Assume now that there would be a certain amount of spatial separation between an "end organ" and the discharging region of its nerve so that the former could be cooled without paralyzing the latter. The resulting structure would be something analogous to the fish *Lorenzinian ampullae* which were found by Sand (1938) to respond to cooling by discharging and to warming by cessation of impulse activity. Their mechanism of excitation may well be of the kind described in this paper.

The applicability of the model is perhaps more striking with respect to cooling. The mechanism is quite sensitive. A quick fall of 1° may cause a discharge in a good preparation. The generation of impulses in response to heat is provided for by the model in the impulse activity which is a consequence of warming the "end organ" above the normal range. Its mechanism is apparently similar to the generation of impulses by cold. The "end organ" again

becomes negative relative to normal nerve. We need but imagine a certain amount of development and specialization of the temperature end organs, based on the general principles described in this paper, to possess a basic theory for how cold and heat excite their receptors to set up impulse activity in the nerve fibres.

#### SUMMARY

Rapid local cooling of mammalian nerve sets up a discharge which is preceded by a local temperature potential, the cooled region being electronegative relative to a normal portion of the nerve.

Heating the nerve locally above its normal temperature similarly makes the heated region electronegative relative to a region at normal temperature, and again a discharge is set up from the heated region.

These local temperature potentials, set up by the nerve itself, are held to serve as "generator potentials" and the mechanism found is regarded as the prototype for temperature end organs.

Our thanks are due to The Rockefeller Foundation for a grant to this laboratory.

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CORRECTION

In Vol. 29, No. 4, March 20, 1946, page 225, line 17, for  $X K\sqrt{T}$  read  
 $X = K\sqrt{T}$ .



# THE EXTRACELLULAR RELEASE OF ECHINOCHROME\*

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## I. INTRODUCTION

Echinochrome, the red coloring matter found in sea urchin eggs, is not uniformly dissolved in the cytoplasm, but is distributed in the form of granules, and may be associated in these formed elements with other cellular components. It may exist, intracellularly at least, as a chromoprotein.

That the echinochrome is not in solution in the unfertilized egg cell, but exists practically entirely in the form of these discrete particles, is evident through microscopic examination and from centrifuging experiments. Under the influence of centrifugal force, the echinochrome granules can be concentrated at one end of the cell where they are easily visible, and with still higher centrifugal force the colored part of the cell can be separated from the remainder (Harvey, 1941; Shapiro, 1935 *a*, 1939). Tyler (1939) has made a survey of some of the chemical and physiological studies on this substance.

If the peristomial membrane of an urchin, whether male or female, be punctured with the point of scissors, and the coelomic (perivisceral) fluid poured into a vial, it is observed to have a deep reddish coloration. Examined microscopically, this is found to be a concentrated suspension of eleocytes, which also contain the echinochrome. In eggs freshly taken from the urchin together with coelomic fluid, the coloring of the supernatant fluid is due to the presence of these coelomic amebocytes. However, in some cases after the eggs have been allowed to settle out of the colored supernatant fluid, the eleocytes are nevertheless found there in but low concentration. It is to be presumed then that the echinochrome has diffused out of the eleocytes, or that part of the color may be due to the contact of the coelomic fluid with the test. It was very likely with such a preparation that Glaser (1921) was dealing in his description of secretions from the unfertilized egg. MacMunn (1885) observed the clotting of eleocytes from the perivisceral fluid of *Strongylocentrotus lividus*.

## 2. Technic, and Observations on Unfertilized Eggs

A large quantity of eggs from one or several urchins was obtained in the customary manner (Shapiro, 1935 *a*). The eggs were made up in concentrated suspensions (see

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\* This investigation was aided by a grant from the Penrose Fund of the American Philosophical Society.

Tables I to IV) in filtered sea water, and a 50 ml. sample poured gently into a 250 ml. Erlenmeyer flask previously equilibrated in a constant temperature water bath at 25.6°C. The cells soon settled to the bottom of the flask and thus lay beneath a shallow layer of sea water. Fertilized eggs were obtained by adding sperm to the suspension, allowing the eggs to settle, pouring off the supernatant, then washing with sea water, and again removing the supernatant after brief light centrifuging of duration sufficient only to throw the cells down. The washed fertilized eggs were introduced into the flask at constant temperature within about a half hour, on the average, after removing the eggs from the urchin. Eggs were selected for experiments only when a test fertilization with freshly suspended sperm showed a high percentage of membranes (usually 99 to 100 per cent). To insure an adequate oxygen supply, oxygen was first moistened by being bubbled through sea water at the temperature of the water bath, and then passed slowly and continuously over the surface of the shallow layer of sea water suspension (not bubbled through, as this might

TABLE I  
*Unfertilized Eggs in Sea Water*

Note that echinochrome loss (tinting of the supernatant fluid) occurred only in anaerobic eggs (Experiments 11 and 18).

Experiment No.	Gas	Cell concentration cells/ml.	Samples withdrawn min.	Echinochrome loss
11	N <sub>2</sub>	1,970,000	20,53,87,139	Tint first noted at 3rd sample
13	O <sub>2</sub>	304,000	39,150,204	No loss
14	O <sub>2</sub>	532,000	31,81,203,347	Supernatant clear
18	N <sub>2</sub> , then O <sub>2</sub> after 3rd sample	411,000	30,64,111,288	Slight tint in supernatant
19	O <sub>2</sub>	519,000	34,81,174,251	Supernatant clear
21	O <sub>2</sub>	579,000	54,96,160,237	Supernatant clear
30	O <sub>2</sub>	237,000	62,170	Supernatant clear

lead to cytolysis). The flasks containing the eggs were kept throughout the experiment at constant temperature, and opened at regular intervals after a slight agitation to completely resuspend the cells, and a sample slowly pipetted out and placed in a centrifuge tube. A standard force and centrifuge time were adopted, and observations were made of the coloring of the supernatant fluid. No attempt was made to compare quantitatively the colorations in the different experiments. Cell counts were made by a modification of the dilution method previously described (Shapiro, 1935 b). Each time a sample of cells was withdrawn for centrifuging, a microscopic examination was made to determine the cytological condition of the cells and, in the case of fertilized eggs, the amount of cleavage. A clean dry pipette was used for each sample.

When unfertilized eggs are thus left in normal sea water for periods as long as 5 hours, as Table I indicates, usually very little cytolysis ensues, and the eggs at the conclusion of the run are fertilizable and undergo normal develop-

ment. The supernatant fluid remained clear in all the experiments listed, showing the retention of echinochrome by eggs in the unfertilized state over this period of time. That is, no coloration was observable on viewing through a column of fluid 15 mm. in diameter. This is a sensitive test, as the eye can detect very dilute concentrations of dyes in solution in such an arrangement. In only one experiment (not included in the table) was any tinting of the supernatant noted for unfertilized eggs in oxygen, and there, in addition to cytolysis, a large amount of cellular debris was suspended as a fine sediment in the supernatant. It appears clear then that normally, unfertilized oxygenated eggs do not release their pigment into the sea water, even though they be left standing for hours.

This retention of pigment by unfertilized eggs does not occur, however, when the cells are made anaerobic by exchanging the oxygen stream for one of nitrogen. The eggs will then part with some of their echinochrome, though it may be at a slow rate and in relatively small quantities. Accordingly, unfertilized eggs left long enough under conditions approximating anerobiosis (e.g., crowded in covered vessels with inadequate oxygen supply) may be expected to lose some echinochrome. Unfertilized cells under anaerobic conditions may glycolyze, producing acid, thus tending to be in a state similar to that of the fertilized *Paracentrotus* egg which according to Runnström (1933) forms acid after both aerobic and anaerobic fertilization. Perlzweig and Barron (1928) observed lactic acid production both in unfertilized and fertilized eggs of *Arbacia punctulata*. In the urchin *Paracentrotus lividus*, Rapkine's (1931) measurements revealed a minimum of lactic acid production shortly after fertilization, a marked increase about the 40th minute, and a maximum about the 50th minute (i.e., 10 to 15 minutes before cleavage).

This acid production may be one of the conditions favorable for the release of echinochrome, and its increasing concentration in the sea water during the first cleavage cycle would then accord with the data given in the ensuing paragraph.

### 3. Diffusion of Echinochrome from Fertilized eggs

Turning now to the fertilized eggs (Table II) we find that *activation in all cases resulted in a release of echinochrome by the fertilized egg*. This release continues over a period of time, for in successive samples the depth of the color will frequently show a progressive increase. The pigment release begins during the mitotic events leading to first cleavage, for eggs examined before any divisions have set in will show echinochrome in the supernatant fluid. Whether this begins immediately upon insemination is not known, as the initial observations were made some time later, but it appears likely that it does. McClendon (1912) observed the migration of the echinochrome granules to the surface within half an hour after fertilization, and their accumulation in the

cleavage furrows during cleavage. And according to Rapkine the lactic acid production increases during the course of the first mitotic division.

The large efflux of echinochrome observed in the case of fertilized eggs cannot be due to cytolysis, as a check was maintained on this factor in all the experiments. For the most part, the amount of cytolysis in the various experiments was very small, and the final microscopic examination of the eggs at the end of the experiment revealed an average of from 1 to 5 per cent cytolysis in the unfertilized eggs. The fertilized eggs suffered even less change, and underwent only 1 per cent cytolysis on the average. In the case of one sample of unfertilized eggs in which a relatively large amount of cytolysis had

TABLE II  
*Fertilized Eggs in Oxygenated Sea Water*

All fertilized eggs release echinochrome.

Experiment No.	Cell concentration cells/ml.	Samples withdrawn min.	Echinochrome loss
1	74,000	9, 18, 27, 40, 61	Yellowish tint, increasing with time
2	49,000	42, 72, 102, 131, 188	Tint in supernatant, progressively increasing
4	320,000	26, 55, 92, 133	Supernatant deeply tinged
6	308,000	9, 42, 68, 258	Supernatant tinted
15	348,000	31, 81, 203, 347, 394	Supernatant tinted
19	519,000	34, 81, 174, 251	Supernatant deeply tinged
23	482,000	43, 94, 165, 319, 359, 443	Supernatant tinted
29	237,000	73	Slight tinge in supernatant
30	237,000	62, 170, 257	Supernatant deeply tinged

occurred there was practically no observable tint in the supernatant fluid; and conversely, in batches of fertilized eggs with practically no cytolysis, a marked tint was present in the sea water.

The diffusivity of echinochrome through the cell membrane before and after fertilization has not been investigated quantitatively. However, the phenomenon under discussion is not simply analogous to the marked change in water permeability which occurs on fertilization (Lillie, 1916; and others) for it involves also a change in the cell interior which brings about a dissolution of echinochrome from the granules to which it is bound.

Certain lots of eggs tend to disintegrate much more readily during the experiment than others. The cellular remains were apparent as a fine silt in the supernatant fluid, after brief centrifuging, evidently more difficult to throw down. The greater tendency of these eggs to disintegrate was due probably to their age and nutritional state, as they were taken from urchins which had been in the aquarium about 4 weeks, without food supply.

#### 4. A Physical Analogue of the Phenomenon

To illustrate the concept developed above, it is instructive to utilize the analogy of the charge and discharge of a condenser (Fig. 1). The condenser  $C$  represents the echinochrome containing granules,  $R_1$  the cytoplasm,  $R_2$  the cell membrane, and the battery  $E$  the chemical embryological processes leading to synthesis of echinochrome and its concentration within the granules. During cell growth and maturation, the switch  $S$  is in position 2, and remains there, even when the eggs are liberated into the sea water. All the echinochrome has been accumulated in the granules. On fertilization (or the institution of anaerobiosis), indicated by the arrow under the graph, the switch is thrown by chemical events over to position 1. The charge (of echinochrome) accumulated in the condenser (granules containing echinochrome-protein complex) is

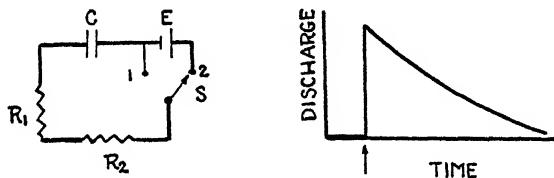


FIG. 1

liberated, encountering two chief barriers, the cytoplasm, and the cell membrane ( $R_1$  and  $R_2$ ). More formally,

$$i = \frac{d}{dt} \left( e^{-t/(R_1+R_2)C} \right) = - \frac{q_0}{(R_1 + R_2)C} e^{-t/(R_1+R_2)C} \quad (1)$$

The analogies in equation (1) are that  $i$  (current) represents rate of flow of echinochrome out of the cell,  $q_0$  (charge) the amount of echinochrome accumulated in the granules, which are the condenser  $C$ ; and  $t$  represents time.

#### 5. The Effect of Changes in the Chemical Content of the Sea Water

Following these results, observations were made on the influence of altered environment. It is known that such an alteration will affect the permeability of the cell (e.g., to water), and may also affect the ionic constitution of the cell interior. Solutions used were those listed elsewhere (Shapiro, 1941) in addition to others made up as follows:—

##### I. Artificial sea water, 2 $\times$ potassium (2 K):

NaCl	0.52 M	978 ml.
KCl	0.53 M	44 ml.
MgCl <sub>2</sub>	0.37 M	78 ml.
MgSO <sub>4</sub>	0.52 M	38 ml.
CaCl <sub>2</sub>	0.34 M	15 ml.

0.5 ml 0.01 N NaOH added to 50 ml. pH 8.71, 26°C.  $\Delta = -1.896^\circ\text{C}$ .

## II. Artificial sea water, 5 X potassium (solution A) (5 K):

NaCl	0.52 M	912 ml.
KCl	0.53 M	110 ml.
MgCl <sub>2</sub>	0.37 M	78 ml.
MgSO <sub>4</sub>	0.52 M	38 ml.
CaCl <sub>2</sub>	0.34 M	15 ml.

0.5 ml 0.01 N NaOH added to 50 ml. pH 8.72, 26°C.  $\Delta = -1.872^\circ\text{C}$ .

## III. Sea water, 5 X potassium (solution B) (5 K, sea water):

Add 88 ml. 0.53 M KCl to 1153 ml. natural sea water (filtered from aquarium tap). pH 7.86, 25.2°C.

## IV. Artificial sea water, 4 X calcium (4 Ca):

NaCl	0.52 M	1000 ml.
KCl	0.53 M	22 ml.
MgCl <sub>2</sub>	0.29 M	33 ml.
MgSO <sub>4</sub>	0.52 M	38 ml.
CaCl <sub>2</sub>	0.34 M	60 ml.

0.12 ml. N/1 NaOH added to 1000 ml. pH 6.96, 27°C.  $\Delta = -1.764^\circ\text{C}$ .

0.22 ml. N/1 NaOH added to 1000 ml. pH 7.27, 25.2°C.

Unfertilized eggs in artificial sea water with excess potassium (Table III) retained their echinochrome. One run with eggs in excess calcium solution showed practically no tinctorial effect on the supernatant. Other substances, however, are known to induce the liberation of the pigment. As McClendon (1909) and E. N. Harvey (1910) have shown, *sufficiently concentrated* parthenogenetic reagents cause the echinochrome to diffuse out of the cell. It is interesting, then, to note that this particular effect of a parthenogenetic agent is similar to that brought about by the normal process of fertilization.

The yellow pigment in the cells of the larva of *Arenicola cristata* was shown by Lillie (1909) to be released, and the embryos caused to contract strongly, when placed in isotonic solutions of NaCl, KCl, and other substances. This was taken by Lillie as evidence of the general increase in permeability induced by these salts.

In the case of the fertilized eggs in modified sea waters (Table IV), the escape of echinochrome occurred in all of the experiments, and here again, the initiation of the pigment release occurred during the first cleavage. The pH of the excess calcium solutions was 6.96 and 7.27 that of the excess potassium, 7.86 and 8.72. It is evident from this that the altered ionic environments were unable to prevent the intracellular change occurring after fertilization, which leads to the diffusion of echinochrome out of the cell.

It is of interest to compare these results with the passage of another cellular constituent, potassium, across the cell membrane. In both fertilized and unfertilized states, potassium (as determined by microchemical analysis) diffuses *out* of the eggs in normal sea water environment (Shapiro and Davson, 1941). When placed in sea water containing five times the normal amount of KCl, potassium diffused *into* both fertilized and unfertilized eggs, against a

gradient (the concentration of K inside the cell is twenty times as great as in the sea water), *i.e.* in the fertilized eggs, at the same time K was entering the cell, echinochrome was diffusing out. In fertilized eggs in *normal* sea water, both K and echinochrome diffused out of the cell. Apart from the diffusivity of echinochrome itself, there is the problem, perhaps related to the intracellular

TABLE III  
*Unfertilized Eggs in Modified Oxygenated Sea Waters*

Experiment No.	Cell concentration cells/ml.	Medium	pH	Samples withdrawn min.	Echinochrome loss
17	365,000	5 K	8.72	57,115,452	Supernatant clear
27	520,000	5 K sea water	7.86	81	Supernatant clear
10	943,000	3 Ca (solution G)	7.02	15,54,258	Practically none; supernatant has eleocytes
22	421,000	4 Ca; then sea water	6.96	54,96,160,237	No tint noted

TABLE IV  
*Fertilized Eggs in Modified Oxygenated Sea Waters*

Experiment No.	Cell concentration cells/ml.	Medium	pH	Samples withdrawn	Echinochrome loss
3	39,300	2 K	8.71	31,48,87,138	Very slight tint
16	555,000	5 K	8.72	57,115,452	Tint present
26	520,000	5 K	7.86	81,142,213	Deep tint
8	670,000	Ca-free sea water	7.22	12,42,84	Very slight tint
9	459,000	3 Ca	7.02	17,43,77,115	Tint present
24	367,000	4 Ca; then sea water	6.96	43,94,165,319,359,443	Tint present
28	237,000	4 Ca	7.27	73,188	Supernatant tinted

acid concentration, of how echinochrome is liberated from its granular combination within the cell. The pigment diffusing from the fertilized eggs requires further investigation as to its chemical nature—whether it is echinochrome, or some echinochrome complex. The developmental significance of the extracellularly released echinochrome has still to be elucidated.<sup>1</sup> Tyler (1939) and Cornman (1941) have found it to have no sperm-activating effect.

<sup>1</sup> After this manuscript had been completed, my attention was drawn to a note by Lyon and Shackell (Lyon, E. P., and Shackell, L. P., On the increased permeability of sea urchin eggs following fertilization, *Science*, 1910, 32, 249) containing a reference to

## SUMMARY

A study was made of the diffusion of the red pigment echinochrome from the eggs of the sea urchin, *Arbacia punctulata*, into sea water.

Unfertilized eggs retained their pigment, over periods of hours. Outward diffusion of pigment from unfertilized eggs normally is entirely negligible, or does not occur at all. Enhancing the calcium or potassium content of the artificial sea water (while retaining isosmotic conditions) did not induce pigment release.

Under anaerobic conditions, unfertilized eggs release pigment in small quantities.

Fertilization alone brings about echinochrome release. Fertilized eggs invariably released pigment, whether in normal sea water, or sea water with increased calcium or potassium. This diffusion of the pigment began during the first cleavage, possibly soon after fertilization.

The pigment release is not a consequence solely of the cell's permeability to echinochrome (or chromoprotein, or other pigment combination) but is preceded by events leading to a release of echinochrome from the granules in which it is concentrated within the cell. These events may be initiated by activation or by anaerobiosis.

The phenomenon was not due to cytolysis.

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the observation that echinochrome was released, and the inference that this was due to a permeability increase. The latter statement requires experimental substantiation, and in the body of the present paper it is indicated that the primary effect is probably a chemical action on the granules.

That the release of echinochrome on fertilization has not been a generally understood and accepted fact is indicated by the contents of at least one much later paper (*Physiol. Zool.*, 1940, **13**, 212) which misses the point that fertilization by itself brings about pigment release from the intact living cell.

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# THE CHEMISTRY OF DAYLIGHT VISION

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## *I. Photopic and Scotopic Vision*

Daylight vision in man is synonymous with color vision, a faculty which is equally prominent in diurnal fish, reptiles, and birds. Mammals other than the primates seem to be color blind and yet may possess the same types of visual cells as color discriminating forms. Color vision thus represents one aspect of a broader system known as daylight or photopic vision. This system, which is functional only at the relatively high intensities of ordinary daylight, is characterized by the high visual acuity it affords and by a spectral visibility curve maximal at  $555 \text{ m}\mu$  in the yellow green (Ives, 1912; Hyde, Forsythe, and Cady, 1918; Coblenz and Emerson, 1917; Gibson and Tyndall, 1923; Stiles, 1944; Pinegin, 1944; and Wald, 1945).

The visibility curve may be defined as the reciprocal of the energy or, more usefully, of the number of quanta required to produce a constant brightness throughout the spectrum. The determination of this curve is complicated by the fact that color differences confuse the estimation of brightness. This difficulty has been minimized by matching adjacent regions of the spectrum step by step (Gibson and Tyndall) or by flicker photometry (Coblenz and Emerson), a method in which advantage is taken of the fact that alternate presentation of two colors at a sufficiently high frequency results in fusion of the colors without disappearance of the flicker due to the difference in their brightness.

If the intensity of spectral light is lowered enough a region is entered in which the light appears colorless. In this range a visibility curve with a maximum about  $500 \text{ m}\mu$  in the blue-green is obtained (Hecht and Williams, 1922). Clearly a different, color blind, system is functioning at these low energies. Known as the scotopic system, it has furnished nearly all our knowledge of the chemistry of visual substances.

## *II. The Chemistry of Scotopic Vision*

The scattered and often contradictory observations on the chemistry of daylight vision require confirmation and extension. Since the kinetic data of vision show a close relation between scotopic and photopic vision in such features as light and dark adaptation, visual acuity, and intensity discrimination (Hecht, 1937), a summary of the salient features of the scotopic system will serve as a useful introduction to this investigation. More detailed in-

formation may be obtained from the recent reviews by Hecht (1942) and Wald (1943).

Scotopic vision is correlated with the presence of large numbers of rod cells in the retina. The rod-rich retinas of the frog were found by Boll in 1876 to contain a red substance which bleaches in the light and reforms in the dark. This substance, known as visual purple or rhodopsin, may be extracted into aqueous solution by surface-active detergents such as the bile salts (Kühne, 1877), digitonin and saponin (Tansley, 1931). The absorption spectrum of purified visual purple (Chase and Haig, 1938; Lythgoe, 1937; Wald, 1938;

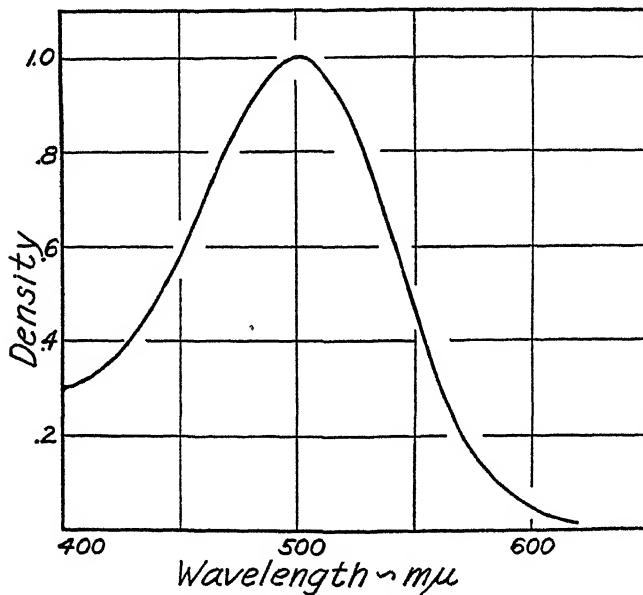


FIG. 1. Absorption spectrum of visual purple (Hecht, 1942). Average of data from three sources (Chase and Haig, 1938; Wald, 1938; and Lythgoe, 1937).

and Krause and Sidwell, 1938) forms an unsymmetric curve with a maximum at  $500 \text{ m}\mu$ . The absorption spectrum of frog's rhodopsin, collected from three sources, is shown in Fig. 1.

Exposure to light results in a fall in absorption maximal at  $510 \text{ m}\mu$  and a rise in absorption toward the violet end of the spectrum. The difference spectrum of visual purple is shown in Fig. 2, B. The rise in absorption in the blue means that rhodopsin bleaches to a yellow photoproduct. The difference spectrum is therefore only approximately similar to the absorption spectrum.

When the scotopic visibility curve is computed as the relative number of

quanta which must reach the retina in order to evoke a given visual sensation (Schneider, Goodeve, and Lythgoe, 1939) good agreement is found with the absorption of the same amount of rhodopsin known to be in the human retina (Wald, 1938; Hecht, Shlaer, and Pirenne, 1942). All these facts demonstrate that rhodopsin is the basis of scotopic vision.

Rhodopsin has the physical and chemical properties of a protein. It is destroyed by heat above 50°C., strong acid or base, and by many polar organic solvents. If an excised retina is illuminated just long enough to bleach it and is then shaken with chloroform, a yellow carotenoid, retinene, is extracted (Wald, 1935). This previously unknown pigment is characterized by an absorption peak at 387 m $\mu$  in chloroform and at 664 m $\mu$  with antimony trichloride. Since oxidation of vitamin A produces a similar chromogen (Hunter and Hawkins, 1944) Morton and Goodwin (1944) suggest that retinene is vitamin A aldehyde. After prolonged illumination retinene can no longer be found. In its place however is a substance absorbing maximally at 328 m $\mu$  with the properties of vitamin A. Rhodopsin therefore appears to be a conjugated protein which liberates a prosthetic group related to vitamin A as a consequence to the absorption of light.

Dark adaptation of the living eye results in the removal of vitamin A and the regeneration of visual purple. Deficiency of vitamin A in the diet results in night blindness due to the failure of visual purple regeneration (Holm, 1925; Fridericia and Holm, 1925; Tansley, 1931). It is significant that the photopic threshold also rises in this condition (Hecht and Mandelbaum, 1938), suggesting that photopic vision likewise involves vitamin A.

### *III. Cone Pigments*

The similarity in the dynamics of the photopic and scotopic systems leads us to look for rhodopsin-like pigments basic to photopic vision. Daylight vision is correlated with the presence in the retina of large numbers of cone cells. These are somewhat similar to the rods but contain no visible photosensitive pigments. Köttgen and Abelsdorff (1896) made glycocholate extracts of the pure cone retinas of tortoises, and Kühne (1877) investigated the chicken retina but in neither animal could any trace of photopigment be demonstrated. The fact that the energy threshold for photopic vision is about 100 times the scotopic level in human beings suggests that one reason for the failures was the low concentration of cone substances. It has therefore become clear that further progress requires the application of high precision spectrophotometric methods to the cone-rich retinas of the diurnal birds, reptiles, or mammals.

In recent years several investigators have reported the extraction of cone pigments from tortoises, chickens, and frogs. The first claim was made by von Studnitz (1937) who reported that ether extracts of tortoise and frog

retinas contained a photosensitive substance with a maximum absorption at 570 m $\mu$ . However, this claim cannot be accepted because the height of this absorption maximum is little greater than the sensitivity of his galvanometer (1 mm. deflection for 0.1 lux). In his figure of the absorption spectrum of cone substance from frogs the absorption peak at 570 m $\mu$  absorbs 0.3 per cent more light than the minimum on the long wave side. Since the total illumination is 4.5 lux, a difference in absorption of 0.3 per cent would cause the scarcely detectable change in deflection of only 0.14 mm. The insignificance of this peak is further emphasized by the fact that von Studnitz ignores the existence of an absorption at 630 m $\mu$  actually greater than that at 570 m $\mu$ .

So much has been made of these findings by von Studnitz (1940) that the experiments seemed worthy of repetition even though Hosoya, Okita, and Akune (1938) and Wald (1943) have already failed to confirm them. An ether extract of frog retinas was prepared following the procedure in his report and was measured on Shlaer's spectrophotometer (1937). The solution yielded only a smooth absorption curve rising into the violet, and showed no photosensitivity.

Hosoya, Okita, and Akune (1938) presented evidence for cone pigments of more conventional properties in saponin and glycocholate extracts of tortoise and frog retinas. They reported difference spectra with several poorly defined peaks supposedly representing bleaching of the cone primary substances by their measuring light source.

In the course of a search for cone substance in the frog, I found a similar fall in the absorption of freshly prepared extracts quite independent of illumination. This fall apparently is due to autolysis of the fresh extract. Since Hosoya and his coworkers present no control data in this regard, their results are open to question.

Wald (1937) reported the extraction of a photosensitive cone pigment from chicken retinas with aqueous digitalin. Since a small quantity of rhodopsin is present in the eyes of chickens he found it necessary to bleach the solution with dim red light beyond 650 m $\mu$ , to which rhodopsin is relatively insensitive. Exposure to red resulted in a decided drop in absorption maximal at 570 m $\mu$  in the yellow, and a rise beginning at about 460 m $\mu$  and extending into the ultraviolet. Evidently the chicken retinas contained an extractable red-sensitive substance which bleached to form a yellow photoproduct. Wald has proposed the name "iodopsin" for this new visual pigment. It is significant that its difference absorption spectrum bears a rough resemblance to the visibility curve of the light-adapted chicken (Honigman, 1921).

After the iodopsin was completely bleached exposure to white light resulted in a further fall in absorption with a maximum at 510 m $\mu$  characteristic of visual purple.

I have been able to reproduce Wald's results in a great many repetitions of

his work. A typical set of curves obtained from an extract prepared as described below may be seen in Fig. 2. While the heights of the difference spectra in this figure have been arbitrarily adjusted to the same height, actually the maximum density fall resulting from the bleaching of iodopsin was found usually to be about  $\frac{3}{4}$  that of rhodopsin.

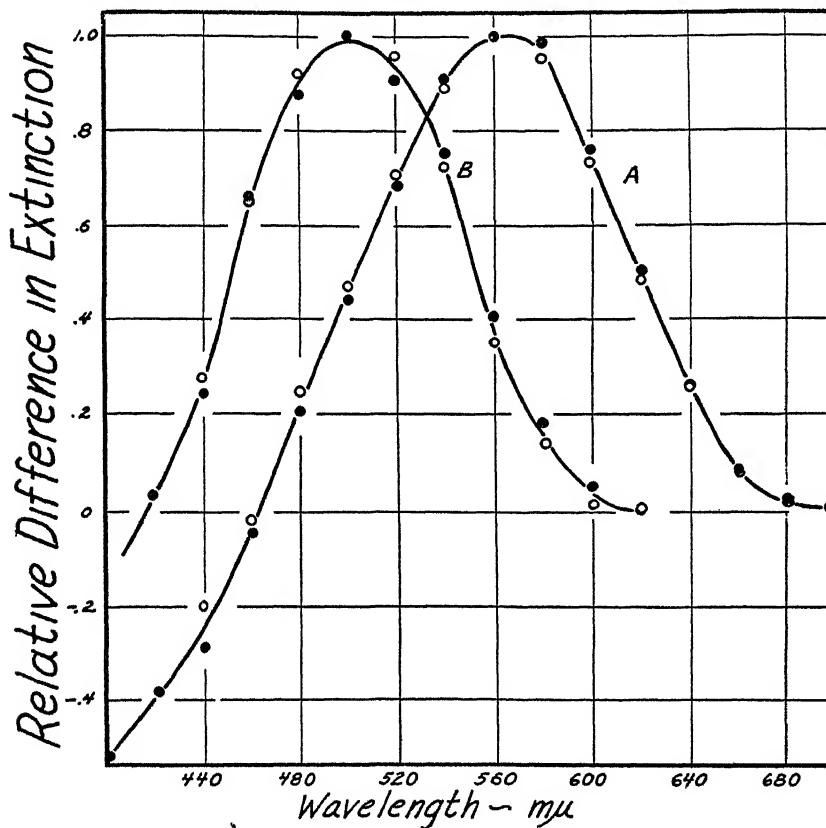


FIG. 2. Difference absorption spectra of photosensitive pigments from chicken eyes. A, change due to red light. B, change due to white light after completion of bleaching by red light.

Wald's discovery is a significant start toward a chemistry of cone vision. It was therefore decided to obtain further information about the photochemical properties of iodopsin.

#### IV. Apparatus

The instrument with which most of this investigation has been carried out is a potentiometric photocolorimeter (Bliss, 1943). Its main features are shown in Fig. 3.

The light of a 32 c.p. 6 volt storage battery-operated automobile headlight bulb is passed through a prism monochromator with a range of 360 to 700 m $\mu$ . The wavelength scale is calibrated with mercury and helium spectra and may be read to the nearest millimicron. The bilateral slits are adjusted to produce an approximately equal sensitivity through the spectrum. Under these conditions the band width varies between 5 and 10 m $\mu$ .

The solution to be measured is contained in a 1 cm. corex D cell of 1 cc. volume which is mounted on a shuttle in a removable light-tight box. The solution may be moved in and out of the beam, permitting measurement of the transmission ( $I_t/I_0$ ). When the shuttle is pushed to the far end of the box, light is admitted for tests of photosensitivity.

Stray light, always a source of error when making density measurements in regions of low energy, could be disregarded above 400 m $\mu$  due to the use of a RCA 926 photo-

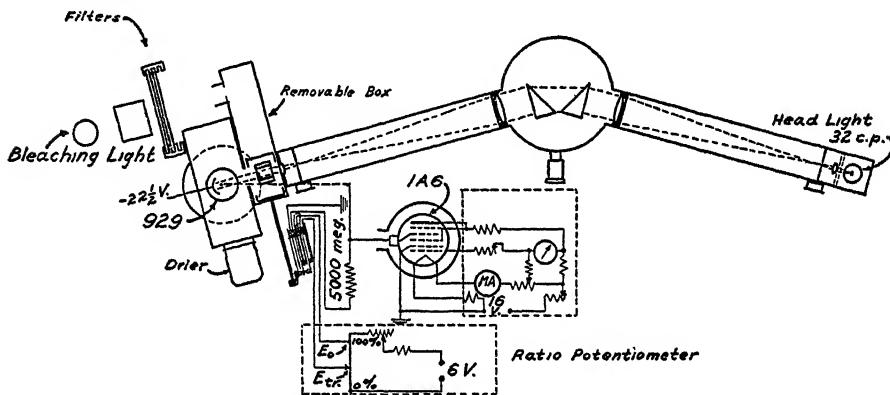


FIG. 3. Plan of spectrophotometer and bleaching lights.

tube with maximum sensitivity in the blue. At present even better response in the blue is attained with the use of a 929 tube with maximum sensitivity at 375 m $\mu$ . The anode lead of this tube is passed through a hole provided by the removal of the center post of the tube base, to reduce insulation leakage. The small energy in the ultra-violet spectrum of the source necessitates the use of a Corning 597 filter in front of the monochromator to cut down stray light of longer wavelengths when measuring densities below 400 m $\mu$ .

The photoelectric amplifier used for this investigation consisted of a 1A6 tube in Barth's modification (Penick, 1935) of a DuBridge and Brown balanced circuit. However, much greater gain and stability is now achieved with a twin triode 6F8, in a bridge circuit. The grid leak consists of a 300 megohm India ink line resistor. A box galvanometer with a sensitivity of 30 mm. per  $\mu$ a is used as a null point indicator with an 11 turn helical potentiometer giving the transmission ( $I_t/I_0$ ) directly. Results usually are expressed as photometric density (-log transmission).

*V. Efforts at Purification of Iodopsin*

(a) *Stability.*—The following observations show that iodopsin bears considerable resemblance to rhodopsin but is much more labile. Unless otherwise noted 4 to 12 retinas per ml. were extracted in 2 per cent digitalin for 4 to 8 hours.

While rhodopsin may be extracted after exposure of the retinas to 4 per cent alum at a pH of 2.9 and is unharmed in solution at pH 10, iodopsin could not be extracted if the retinas were previously soaked for 1 hour in 0.1 M acetate buffer below pH 4.4. Full yield was not obtained below pH 4.9. Extraction at pH 8.5 yielded only a trace of iodopsin. Unbuffered extracts kept for 2 weeks at 5°C. yielded the difference spectrum of rhodopsin alone.

Iodopsin could not be extracted by 4 per cent sodium glycocholate or salicin and was destroyed in about 2 hours at room temperature by addition of an equal amount of 4 per cent sodium desoxycholate to a 2 per cent digitalin extract. It is significant that most of the earlier workers used sodium glycocholate in their unsuccessful attempts to extract iodopsin.

Whereas rhodopsin is precipitated unbleached by the addition of formalin to make a concentration of 10 per cent or by the addition of an equal amount of acetone in the cold, iodopsin is destroyed under the same conditions.

As in the case of rhodopsin it proved possible to dry iodopsin *in vacuo* at 0° and to redissolve it largely without destruction by grinding with sand in 2 per cent digitalin.

Both iodopsin and rhodopsin could be extracted from retinas hardened by soaking in 25 per cent saturated lead acetate followed by rinsing with Ringer's solution. No iodopsin could be extracted when fully saturated lead acetate was used.

(b) *Purification.*—The purification of iodopsin was attempted with a view to determining its absorption spectrum. Baby chickens were decapitated, and the eyes removed. Crude digitalin extracts were prepared by slicing 12 to 24 eyes in half with a razor blade in dim red light, scooping out the retinas and adhering pigment layer into 2 per cent digitalin, extracting for 8 hours, and centrifuging clear. Such extracts contained large amounts of inert yellow impurities. These could be partly eliminated by soaking the half eyes in frog Ringer's solution for 1½ hours to split off the black pigment layer. Mammalian Ringer's did not have this action. Soaking was followed by hardening in pH 4.9 acetate buffer for one-half hour. The retinas were then extracted 2 to 8 hours in 1.5 ml. of 2 per cent digitalin.

The ratio of iodopsin to rhodopsin was changed from  $\frac{3}{4}$  to about 6 if the Ringer-soaked retinas were extracted  $\frac{1}{4}$  to 2 hours with 4 per cent Merck's "pure" saponin in  $\frac{3}{4}$  saturated magnesium sulfate, followed by filtration to separate the retinal residue. The yield of iodopsin was then about 50 per cent

of that found in a crude extract. Unfortunately Merck's saponin became unavailable before a method of purification could be perfected. Several other brands of saponin, all of "purified" grade were tested, but proved far inferior in color and activity.

At best extracts made with magnesium sulfate possessed large amounts of impurities. Preliminary hardening with acetate could not be resorted to because such treatment rendered both iodopsin and rhodopsin insoluble.

(c) *Dark Adaptaion*.—Scotopic dark adaptation, due to the regeneration of rhodopsin, is much slower than photopic. Since rhodopsin is a contaminant of iodopsin extracts, an attempt was made to eliminate rhodopsin by extracting retinas from light-adapted chicks after a minimum of dark adaptation.

This was accomplished by placing the chicks for about 15 minutes in a white-lined box with six 100 watt bulbs, removing the chicks individually to the dark, rapidly killing them, excising the eyes, and immersing them in 2 per cent digitalin. The total time in the dark to perform these operations was 2 to 3 minutes. After about 6 hours' extraction the collected retinas were centrifuged down and the extracted iodopsin and rhodopsin determined.

The ratio of iodopsin to rhodopsin was found to be substantially unchanged, and the total amount of each was about  $\frac{3}{4}$  of the amount extracted from animals dark-adapted several hours. It is thus evident that regeneration of photopigments in the living chick was too rapid for differentiation among the component pigments.

(d) *Distribution in the Retina*.—Since cone vision is associated with high visual acuity, a faculty best developed in the retinal fovea, it seemed possible that extracts of the more central part of the retina might contain relatively less rhodopsin than the whole retina. An extract was therefore prepared from eyes sliced  $\frac{1}{4}$  of the distance from the posterior pole instead of equatorially as usual. Inasmuch as the chicken retinal fovea is poorly developed it is not surprising that the foveal portion delivered the same proportion of rhodopsin to iodopsin as usual; i.e., 4:3.

(e) *Extraction of Lizard Eyes*.—The presence of rhodopsin in chicken eyes emphasized the desirability of using a pure cone eye as a source of iodopsin. The diurnal lizards approach this ideal much more closely than chickens.

Fifty retinas of the fence lizard, *Sceloporus*, from animals ranging in size between 6 and 10 inches long were extracted with  $1\frac{1}{2}$  ml. of 2 per cent digitalin for 8 hours. No trace of photosensitivity could be detected in the extract.

Since the *Sceloporus* retinas were only about 0.1 the area of thick retinas, it was decided to try the night lizard, *Xantusia*. Although a nocturnal form, it contains no rhodopsin, relying on enormously enlarged cones for the necessary sensitivity in dim light (Walls, 1942). Eighteen eyes of a small species from the Mojave desert were extracted as above. On the basis of Walls' figures of retinal cross-sections about 10 times as much iodopsin would be ex-

pected from the *Xantusia* extract; nevertheless the extract again contained no photopigments.

(f) *Embryology*.—It was of interest to investigate the stage of development at which iodopsin and rhodopsin appear, on the chance that they might be separated in this way.

The eyes of 6 chicks were removed at 12, 16, and  $18\frac{1}{2}$  days of incubation at 38–39°C., extracted for 8 hours in  $1\frac{1}{2}$  cc. of 2 per cent digitalin, and tested for the presence of iodopsin and rhodopsin. Both substances were present in about equal quantity only in the  $18\frac{1}{2}$  day extraction. It thus is evident that visual substances are first formed on the 17th day of incubation.

#### *V. The Effectiveness of the Spectrum in Bleaching Iodopsin*

While a pure solution of iodopsin has not yet been obtained, a bleaching spectrum can be arrived at even with an impure solution because it is possible to correct for the effect of the impurities. We have seen that the bleaching effectiveness spectrum of rhodopsin resembles closely its absorption spectrum. In view of the similarities in the properties of iodopsin and rhodopsin this identity probably also holds for iodopsin. The relative bleaching effectiveness of light of various colors and known energy was therefore determined for dilute, 6 to 8 hour extracts of iodopsin prepared from acetate-hardened retinas as already described in the section on purification.

##### *(a) Apparatus.—*

Spectral bands were isolated with Corning and Wratten filter combinations. The bleaching light source for points above 450 m $\mu$  was a 100 watt projection bulb run on a 110 volt A.C. voltage stabilizer and placed 6 inches from the 1 cm. corex D cell. The color temperature of this light was determined by an Eastman meter to be 2800°K. All filter combinations used with this source included a Bausch and Lomb heat-absorbing filter.

The bleaching source for points below 450 m $\mu$  was a G. E. mercury arc lamp, type S4, run on 110 volts A.C. The mercury arc lines were isolated by Corning filters in combinations recommended by the manufacturers. A 1 cm. layer of 5 per cent copper chloride solution was placed in the beam to remove the infrared spectrum of the mercury arc. The energy value of each line was determined with an 8 junction Hardy thermopile and a Leeds and Northrup HS galvanometer giving a sensitivity of 0.5  $\mu$ v./mm. Deflections ranging from 2.4 to 25.0 mm. were obtained.

##### *(b) Calculation of Filter Energy Transmission.—*

The transmission ratio ( $E_{tr}/E_o$ ) spectrum of each filter set in the projection bulb series was measured on Shlaer's spectrophotometer. The energy transmission ( $\Sigma E_{tr}$ ) of each filter set is equal to the value of the area under the curve of  $E_{tr}$ , plotted against wavelength.  $\Sigma E_{tr}$  is arrived at by the multiplication of the filter transmission ratio ( $E_{tr}/E_o$ ) at each wavelength by the corresponding value of the energy distribution

( $E_o$ ) in the spectrum of a black body (Skogland, 1929) at the color temperature (2800°K) of the bleaching source. The  $E_{tr}$  curves are shown in Fig. 4.

The center of gravity of the area ( $\Sigma E_{tr}$ ) under each of these curves was determined physically after the curves were cut out of cardboard; the wavelength of this point was used as the approximate equivalent wavelength of the spectral band transmitted by the filter combination. After the bleaching spectrum was obtained each energy curve was multiplied by the bleaching spectrum to obtain a center of gravity closer to the effective wavelength. This correction amounted to a few millimicrons at most in regions where the slope of the bleaching spectrum was steep. It expresses the fact that the most effective part of the filter band is that transmitting the greatest amount of energy absorbed by the photopigment.

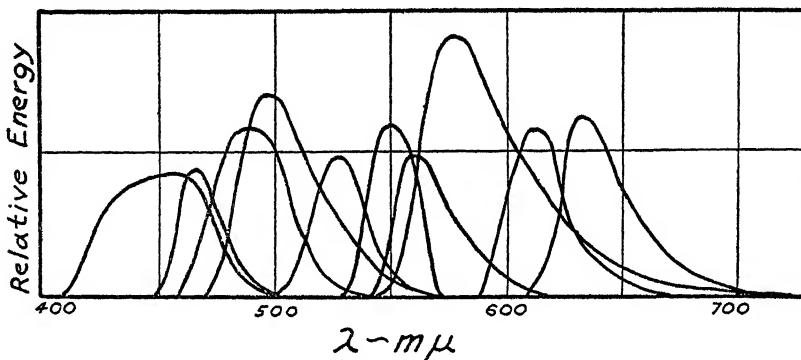


FIG. 4. Energy distribution of filter combinations given in Table 2. Ordinates of curves for filters with central wavelengths of 636, 612, 585, and 450  $m\mu$  are half the scale of the other curves.

Since the absorption of the solution itself contributes to the total filtering, the transmission at the wavelength of bleaching was measured at the end of each bleaching run. After correction for the absorption of the cell and water the value of the transmission at the center of the cell was computed and multiplied by the transmission of the filter set to obtain the total effective energy. Except at 365 and 400  $m\mu$  the absorption of the cell and water could be ignored, since it changed less than 2 per cent about 400  $m\mu$ . Because the energies in the two series of wavelengths were determined independently the bleaching rates could not be directly compared. This relation was achieved by use of the fact that the rate at about 546  $m\mu$  could be measured both in the mercury arc and the projection bulb series. The ratio for the rates found for the two sources at this wavelength was then used as a factor to scale the mercury arc to the projection bulb series.

(c) *Kinetics of Bleaching.*—The bleaching course was followed at 615  $m\mu$  since the maximum absorption fall due to simultaneously bleaching rhodopsin was only about 4 per cent of that due to iodopsin. A rough correction of the bleaching spectrum of iodopsin between 470 and 530  $m\mu$  was made for this

small effect in terms of the fact that rhodopsin was found to bleach about  $1\frac{1}{2}$  times as fast as iodopsin in green light with a dominant wavelength of  $530 \text{ m}\mu$ .

Bleaching was found to be first order as in the case of rhodopsin (Hecht, 1920; Dartnall, Goodeve, and Lythgoe, 1936). This agrees with expectation for a photochemical reaction limited by light intensity. The integrated form of the first order equation assumes the following form in this case:

$$k = \frac{\log a - \log x}{t},$$

where  $k$  is the bleaching factor, proportional to rate,  $a$  is the original concentration of iodopsin, and  $x$  is the concentration of iodopsin at time  $t$ . Expressed in terms of photometric density this becomes:

$$k = \frac{\log (D_0 - D_\infty) - \log (D_t - D_\infty)}{t}.$$

where  $D_0$  is the density before bleaching,  $D_t$  is the density after bleaching for time  $t$ , and  $D_\infty$  is the density after complete bleaching.

In the later stages of bleaching deviations from straightness usually appeared probably because of regeneration of iodopsin, which often reached 10 per cent of the original concentration. The regeneration was evident as a decrease in transmission on successive readings of the density. It gradually declined to zero in the course of several successive bleaches, each of sufficient intensity to cause full bleaching in about a minute. The methods found successful in stopping the regeneration of rhodopsin (addition of pH 11 buffer and extraction with sodium desoxycholate) could not be used because they resulted in the destruction of iodopsin. A number of typical bleaching curves are shown in Fig. 5.

The effect of temperature on the bleaching of iodopsin must be considered. Hecht (1921) showed that the bleaching of rhodopsin, like other photochemical processes, was independent of temperature between 5 and  $35^\circ\text{C}$ . Although it would be surprising to find iodopsin acting differently in this regard, the rate of bleaching of iodopsin in green light of  $546 \text{ m}\mu$  was measured not only at room temperature as usual, but also in one run at  $5^\circ$ . The rates differed by less than 10 per cent—well within the scatter of successive measurements of the bleaching rate under identical conditions.

In order to speed the measurement of the bleaching efficiency the slope of the straight line passing through the points of original and about half-concentration on the logarithmic bleaching curve was taken as the bleaching rate. This determination entailed the following measurements: density before bleaching, at the time of about half-bleaching, and at complete bleaching. At the end of the experiment another density reading was made at the wavelength of bleaching to obtain the self-filtering transmission of the solution.

The density after bleaching was obtained as rapidly as possible to minimize the amount of regeneration during the readings. After the reading was roughly set the solution was again bleached in order to remove the iodopsin and rhodopsin forming during the preliminary reading.

(d) *Light Intensity and Rate of Bleaching.*—Before making the final calculation of bleaching effectiveness one further fact must be established—the linearity of the relation between rate of bleaching and incident energy. The nature of this relation was tested with the aid of the inverse square law.

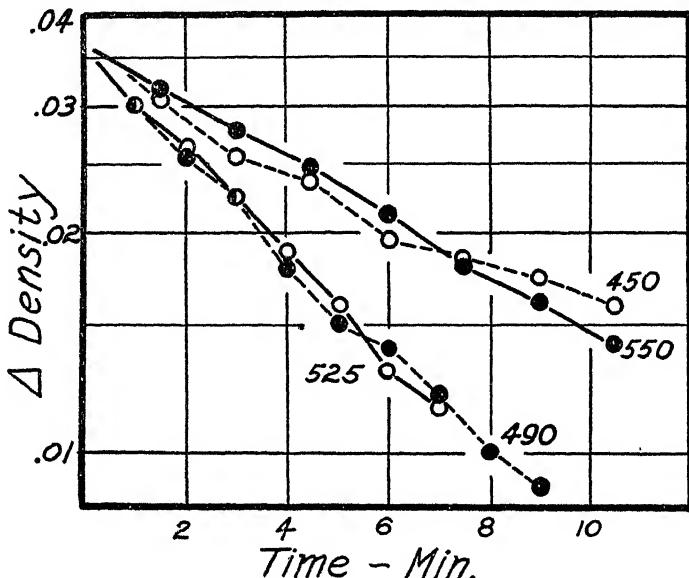


FIG. 5. Kinetics of bleaching of iodopsin with four parts of the spectrum. The density of the solution is measured at 615 m $\mu$ .

A 32 c.p. 6 volt automobile headlight, run at 5 volts A.C. was placed successively at 14.14 and 100 cm. from the center of the cell and the rate of bleaching at the two distances compared. Under these conditions the half-times of bleaching fell within the range used for spectral bleaching; *i.e.*,  $\frac{1}{2}$  minute to  $\frac{1}{2}$  hour.

A 2 per cent digitalin extract was prepared containing about 0.007 density per cm. of iodopsin at 615 m $\mu$ . Samples of this extract were bleached about 50 per cent in times inversely proportional to the energy of the incident light. The bleaching effectiveness of the two energies was calculated from the following equation:

$$\text{Bleaching effectiveness} = \frac{\log (D_0 - D_\infty) - \log (D_t - D_\infty)}{(t) \text{ (energy)} \text{ (wavelength)}}$$

The values actually obtained at the two relative energies are given in Table I. They show that the bleaching effectiveness is independent of the energy used

TABLE I  
*Light Intensity and Exposure in Bleaching of Iodopsin*

Relative energy	Time	Bleaching effectiveness		
		1	2	Average
0.5	25.0	0.0194	0.0179	0.0187
25.0	0.5	0.0187	0.0187	0.0187

TABLE II  
*Relative Effectiveness of Spectrum in Bleaching Iodopsin Projection Bulb Series*

In filter combinations a number corresponds to a Corning glass of standard thickness unless followed by ( $\frac{1}{2}$  S) which means half the standard thickness. A number preceded by (W) represents a Wratten filter from Eastman Kodak. A Bausch and Lomb heat-absorbing glass is designated by H.A.

Filter combinations . . . . .	5113( $\frac{1}{2}$ S) 3389 H.A.	5113( $\frac{1}{2}$ S) 3387( $\frac{1}{2}$ S) H.A.	(W)75 9780 H.A.	3385 3387 5030 H.A.	(W)74 4303( $\frac{1}{2}$ S) 3780 H.A.	3484 4303 5120 H.A.	4303 3482 4407 H.A.	3480 3482 4407 H.A.	2424 2424 4780 H.A.	2408 9780( $\frac{1}{2}$ S) H.A.
Central wave length, m $\mu$ . . . . .	450	479	496	511	532	550	568	585	612	636
Relative energy. . . . .	1440	372	465	1060	416	435	380	2694	1130	1300
Bleaching effectiveness. . . . .	20 16 16 15 21	23 22 22 22 39	44 43 42 40 50	58 57 56 52 50	79 79 77 73 68.5	91 90 90 90 88	99 96 89 85 84	63 63 60 59 59	41 37 36 33 33	15 14 14 14 33
Average effectiveness. . . . .			38	50		87 86.5 85 84.5 80 80 79 78	83	58	57 55 55	
Quantum effectiveness. . . . .	21	25	46	58	77	85	88	59	36	12

for bleaching. The above equation can therefore be used to calculate the bleaching effectiveness of the spectrum if the energy at each wavelength is

TABLE III  
*Relative Effectiveness of Spectrum in Bleaching Iodopsin Mercury Arc Series*

Corning filters . . . . .	{	738 5860	3060 4308 5970	3389 5113	3484 4303 5120
Central wave length, $m\mu$ . . . . .		365	405	436	546
Relative energy . . . . .		6.9	3.7	22.9	9.1
Bleaching effectiveness . . . . .	{	7.4 11.9 12.2 10.2	14.7 10.8 11.2 11.7	15.2 16.0 13.5 18.4	84 84 84 84
Average effectiveness . . . . .		10	12	16	84
Quantum effectiveness . . . . .		15	16	20	84

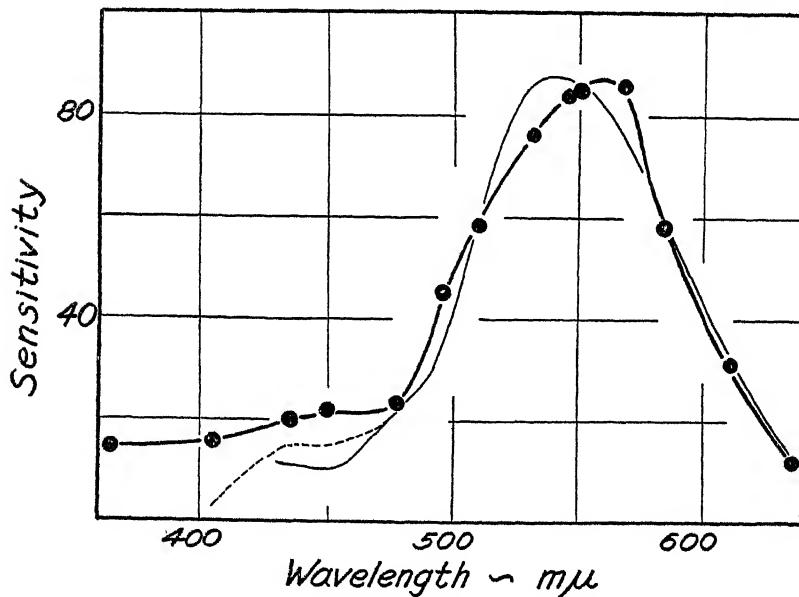


FIG. 6. Relation between spectrum luminosity and spectral sensitivity of iodopsin. Heavy line, spectral photosensitivity of iodopsin. Light line, spectral sensitivity of human cone vision according to Gibson and Tyndall (1923). Dotted line, according to Stiles (1944).

known. Introduction of the (wave length) term in this equation gives us the bleaching effectiveness in terms of quanta and thus makes possible a direct prediction of the corresponding absorption spectrum of iodopsin.

(e) *The Effectiveness Spectrum of Iodopsin and the Photopic Visibility Curve.*—The bleaching effectiveness of the spectrum was computed from the above equation. Because of the variations due to the cumulative effect of the sepa-

TABLE IV  
Photopic Visibility Spectrum

Corrected for ocular and macular transmission and for quantum effect.

<sup>1</sup> Wave length	<sup>2</sup> Gibson and Tyndall luminosity	<sup>3 = 2/1</sup> Quantum luminosity	<sup>4</sup> Ocular trans- mission	<sup>5 = 3/4</sup> Retinal luminosity	<sup>6</sup> Transmission of Macula	<sup>7 = 5/6</sup> Cone luminosity
400	0.5*	0.125	0.086	1.45	0.703	2.07
10	1.2*	0.293	0.106	2.76	0.703	3.93
20	2.2*	0.524	0.160	3.27	0.703	4.66
30	3.3	0.767	0.248	3.09	0.703	4.40
40	4.3	0.977	0.318	3.07	0.703	4.37
50	5.1	1.13	0.388	2.92	0.705	4.14
60	6.9	1.50	0.426	3.52	0.710	4.96
70	10.3	2.19	0.438	5.00	0.718	6.97
80	14.3	2.98	0.458	6.50	0.733	8.87
90	19.6	4.00	0.481	8.31	0.751	11.07
500	31.8	6.36	0.495	12.85	0.781	16.45
10	52.3	10.25	0.511	20.07	0.834	24.06
20	73.2	14.08	0.525	26.81	0.890	30.13
30	87.8	16.57	0.542	30.56	0.924	33.08
40	96.4	17.85	0.552	31.94	0.949	33.65
50	99.8	18.14	0.567	32.00	0.966	33.13
60	99.1	17.70	0.572	36.94	0.979	31.60
70	94.7	16.61	0.583	28.50	0.990	28.78
80	86.3	14.88	0.594	25.05	0.995	25.17
90	75.4	12.78	0.602	21.23	1.000	21.23
600	63.4	10.57	0.610	17.32	1.000	17.32
10	51.1	8.38	0.621	13.49	1.000	13.49
20	38.9	6.27	0.631	9.94	1.000	9.94
30	27.9	4.43	0.640	6.92	1.000	6.92
40	18.4	2.87	0.649	4.43	1.000	4.43
50	11.3	1.74	0.658	2.64	1.000	2.64
60	6.4	0.970	0.664	1.46	1.000	1.46
70	3.5	0.522	0.677	0.771	1.000	0.771
80	1.8	0.265	0.690	0.384	1.000	0.384
90	0.9	0.130	0.696	0.187	1.000	0.187
700	0.4	0.057	0.705	0.081	1.000	0.081

\* Extrapolated.

rate measurements involved in each determination of a bleaching effectiveness, values were obtained from several samples at each wavelength. The individual determinations are given in Table II and Table III, and the averages

are plotted as the bleaching spectrum in Fig. 6. The sensitivity to light is seen to be greatest at 560 m $\mu$ .

In the same figure is the sensitivity of the human cones computed by Hecht (Table IV) from the original luminosity curve of Gibson and Tyndall (1923) corrected for quanta (Dartnall and Goodeve, 1937), for the absorption of the ocular media (Ludvigh and McCarthy, 1938), for the absorption of the macula lutea averaged from the data of Sachs (1891) and Kohlrausch (1931).

Since the values of luminosity below 430 m $\mu$  in Gibson and Tyndall's curve are only roughly checked extrapolations, I have also computed the cone luminosity in the blue from a graph of the photopic visibility curve recently published by Stiles (1944). This is shown as the dotted line of Fig. 6.

The fact that the light sensitivity of iodopsin differs only slightly from that of the human eye throughout nearly the whole visible spectrum is a strong indication of the similarity of iodopsin in the chick to the still unknown cone pigments of the human retina. The deviations, particularly the position of the maxima, may indicate a slight specific difference in the constitution of iodopsin, or the inadequacy of the correction for macular absorpt on, or a difference in the absorption spectrum of iodopsin attendant on solution with digitalin.

(f) *Estimation of Yellow Impurities.*—If we make the reasonable assumptions that the maximum heights of the difference spectra resulting from the bleaching of iodopsin and rhodopsin are approximately equal to the maximum absorptions of these substances, and if it is further assumed that the bleaching effectiveness spectrum of iodopsin is equal to its absorption spectrum, these absorption spectra may be added together and the result compared with the absorption of an actual extract. Perhaps 80 per cent of the absorption at 400 m $\mu$  is thus found to be due to yellow impurities.

#### VI. *Iodopsin and Color Vision*

Following the Young-Hemholtz tri receptor theory, one may assume that there are three types of cones in the human retina, each type containing a photosensitive pigment with an absorption spectrum slightly different from the other two (Hecht, 1934). An alternative theory, suggested particularly for the birds and reptiles, holds that the three types of receptors may be achieved by means of colored oil drop filters in the basal portion of the cones (Wald, 1937). However, Walls (1942) points out that the variable distribution of these drops in the retina indicates that their function is probably the reduction of chromatic aberration rather than color discrimination. Furthermore, Hamilton and Coleman (1933) have shown that the spectral hue discrimination curve of the pigeon is of nearly the same type as that of man, in whose cones oil droplets are lacking.

It would be interesting to test the homogeneity of iodopsin by partially

bleaching it with red or blue light before determining the efficiency spectrum. If it is composed of several primary components, one of the primaries should bleach faster than the others, yielding an efficiency spectrum slightly different from that of the unbleached extract. Unfortunately the scatter of the determinations is too great to justify a search for the small differences to be expected from this treatment.

Weigert has attacked the problem of color vision from a different standpoint. He and his coworkers have shown that rhodopsin in thin gelatine films is photodichroic (Weigert and Morton, 1940). This is an expression of the fact that rhodopsin under these conditions bleaches maximally at the wavelength of the light with which it is bleached. He has suggested that rhodopsin is present in the cones in a similar physical state and therefore affords a mechanism for color discrimination.

However, it is difficult to reconcile such a mechanism with Müller's doctrine of nerve response. Furthermore, the discovery of iodopsin renders Weigert's hypothesis unnecessary.

### VII. Phosphate Production and Illumination

The photochemical bleaching of iodopsin and rhodopsin is only the first step in the visual process. Photoreception must be linked to the metabolic activity of the retina. Dittler's report (1907) of acid production by illuminated frog retinas was an early step in this direction. Later Lange and Simon (1922) showed a phosphate production by illuminated retinas. The frequency with which the work of these investigators is quoted makes desirable a re-examination of the retinal "phosphoric acid production" by modern methods.

(a) *Acid Production.*—The acid production was first investigated by Dittler's method. Each of a pair of *Rana pipiens* retinas, prepared in dim red light, was placed in a 0.5 cc. portion of an indicator solution containing 0.04 gm. NaOH and 0.016 gm. phenolphthalein per 500 cc. unbuffered Ringer's solution. One retina was illuminated with diffuse daylight; the other served as a dark control. The relative amount of fading of the indicator due to acid production by the retinas was observed after 10 to 20 minutes. The pH was then checked by the glass electrode.

Ten repetitions of this experiment showed only a random fluctuation of 0.2–0.7 pH, evidently due to failure, as in Dittler's case, to wash the retinas free from tissue buffers.

The experiment was therefore repeated 5 times with retinas washed 1 hour in 5 to 10 changes of unbuffered Ringer's solution. The pH of such retinas remained constant within 0.2 pH, the limit of colorimetric error of Dittler's method. The positive results reported by Dittler thus probably were due to irregular diffusion of tissue buffers into the alkaline solutions he used.

(b) *Inorganic Phosphate.*—Inorganic phosphate production on illumination

was next investigated, using the Küttner-Lichtenstein (1930) colorimetric method, entailing the reduction by  $\text{SnCl}_2$  of phosphomolybdic acid to blue molybdic oxides. A Bausch and Lomb Duboscq type microcolorimeter with a red filter was used, permitting the measurement of 0.0002 mg. P from a 1 cc. sample with a variability of less than 5 per cent.

Retinas from two dark-adapted frogs were prepared in dim red light and a pair placed in each of two 1 cc. portions of P-free Ringer's solution which was changed 5 to 10 times in an hour to remove free phosphates. The solutions were then changed and their phosphate content determined at 30 minute intervals. One set of retinas remained in the dark throughout the experiment; the other remained in the dark for 30 minutes, after which it was illuminated by diffuse daylight for 30 minutes, and then replaced in the dark.

TABLE V  
*Phosphorus Release in Retina*

Data are given as ratio of experimental to control retinas.

Retina from frogs	30 min. dark	30 min. light	30 min. dark
2 <i>pipiens</i>	0.9	1.1	1.0
2 "	1.9	1.5	1.1
2 "	1.3	1.1	1.1
4 "	1.4	1.1	1.0
4 "	1.4	1.3	1.3
1 bullfrog	0.8	0.7	1.2
2 "	0.7	0.7	1.0
1 "	0.8	0.9	0.8
1 "	1.0	1.3	1.0
1 "	0.8	1.1	1.1
Average.....	1.1	1.1	1.1

The P content of such solutions ranged from 0.0002 to 0.0004 mg. In order to minimize the effects of individual variation and deterioration, the phosphate was expressed as ratio of phosphate produced by the illuminated retina to that of the control. Since individual differences in the ratios are large, the data of 10 experiments are presented in Table V. It is apparent that the present results agree with those of Wald, who has also been unable to confirm Lange and Simon's work. Wald (personal communication) says that his investigation of the balance of organic and inorganic phosphate fractions in the frog retina demonstrates no definite light effect. Lange and Simon give no details of their nephelometric method, so one is at a loss to discover the reason for their results.

The concept of light-induced phosphoric acid production has been accepted and extended by von Studnitz and his coworkers. Their measurements,

however, cannot be accepted because of their failure to perform control experiments. Moreover, the data of Nover (von Studnitz, 1940) demonstrating the spectral sensitivity of retinal acid production are contradicted by those of Wigger (1937) showing the same change in the dark.

It is therefore concluded that there is as yet no convincing demonstration of a light-induced phosphoric acid release from the retina.

#### SUMMARY

1. While several reports of photosensitive pigments from the retinas of animals possessing large numbers of cone cells have been published, the only study which could be confirmed was Wald's discovery of iodopsin, a red-sensitive pigment from chicken eyes.

2. In its chemical properties, such as the range of pH stability and the effect of polar organic solvents, iodopsin resembles rhodopsin but is considerably more labile.

3. A partial purification from inert yellow impurities has been effected by prehardening the retinas in pH 4.9 acetate buffer before extraction by 2 per cent digitonin. Rhodopsin was an inevitable contaminant in most methods of extraction, but could be reduced to about 10 per cent of the absorption due to iodopsin by extraction of unhardened retinas with 4 per cent Merck's saponin in  $\frac{3}{4}$  saturated magnesium sulfate for about 1 hour.

4. The rate of bleaching of iodopsin was found to be first order and linear with respect to energy.

5. The bleaching effectiveness spectrum of iodopsin was determined with the aid of color filters of known energy transmission, and shows a maximum at  $560 \text{ m}\mu$  in the yellow green with a lower plateau in the blue. The spectrum is in good agreement with the sensitivity of the human cones except for the wavelength of maximum bleaching effectiveness. The maximum sensitivity of the human cones is found at  $540 \text{ m}\mu$ .

6. Previous reports of changes in pH and inorganic phosphate level of retinas due to bleaching could not be confirmed.

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# PHOTOLYTIC LIPIDS FROM VISUAL PIGMENTS\*

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Kühne reported in 1878 that visual purple, the light-sensitive protein of the vertebrate retinal rods, bleached in the light with the formation of a pigment which he named "visual yellow." Wald (1935) showed that the yellow pigment of bleached frog retinas could be extracted into chloroform and possessed a maximum spectral absorption at about 385 m $\mu$ . When treated with the Carr-Price reagent for carotenoids, SbCl<sub>3</sub>, a blue color due to an absorption band at 664 m $\mu$  appeared. Wald has named this previously unknown carotenoid "retinene." Hunter and Hawkins (1944) and Morton and Goodwin (1944) have shown that the oxidation of vitamin A leads to a similar chromogen, and the latter authors therefore suggest that retinene is vitamin A aldehyde.

Comparable advances have not been made in the study of daylight vision, the chemistry of which may be regarded as originating in 1937 when Wald reported the existence of a red-sensitive pigment, iodopsin, in the cone-rich retinas of chickens. Its difference absorption spectrum shows a maximum at about 570 m $\mu$  in digitonin solution, near the maximum sensitivity of the light-adapted chicken. This suggests that iodopsin is the basic light-sensitive substance of daylight vision.

The view that rhodopsin (visual purple) is a carotenoid-protein receives support from the decreased sensitivity of the eye to dim light in cases of vitamin A deficiency. Since the thresholds of both night and color vision are raised in experimental vitamin A deficiency (Hecht and Mandelbaum, 1938) it is of interest to determine whether iodopsin likewise releases retinene on exposure to light.

## *Preparation of Retinas*

Iodopsin possesses to an even greater degree the protein-like lability of rhodopsin (Bliss, 1946). It was therefore helpful to find that iodopsin could be preserved indefinitely by freeze-drying a mass of retinas in a vacuum desiccator. This consisted of a two-section bent 1 inch diameter glass tube, one end of which contained a thin layer of retinas, while the other end was immersed in a dry ice freezing bath and thus acted as a condenser. Alcohol at dry ice temperature was poured over the vaporizer

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while pumping with a hyvac pump. The stopcock to the hyvac was then closed and the retinas allowed to desiccate in the frozen state. A charge of about 50 retinas of 1 week old cull chicks could be dried in about 4 hours, yielding 0.4 gm. of friable powder. After extraction of the retinal oils with petroleum ether the powder was stored over drierite in the dark at 5°C.

#### *Experimental Procedures*

Since iodopsin is so much more sensitive to red light than rhodopsin the effect of red light on the free lipid content of the retinal powder could be taken as a measure of the lipid released by iodopsin. 100 mg. samples of powder were mixed with 5 ml. of water in a Syracuse dish mounted in a light-tight box. A Wratten No. 70 (red) filter was placed over the dish. A shallow glass dish holding a 1 cm. layer of water was placed over the filter to absorb the heat of the bleaching light, a 100 watt frosted bulb about 3 inches from the powder.

Before each bleach the suspension was bathed for  $\frac{1}{2}$  hour in flowing CO<sub>2</sub>. After illumination either with 10 minutes of red or 1 to 5 minutes of yellow light (Corning No. 3389), the following operations were performed as rapidly as possible: the retinal suspension was washed into a 1 inch diameter test tube, evacuated a moment to remove trapped gas, and centrifuged 1 minute at a low speed. The supernatant was discarded and replaced with 10 ml. of 2 per cent alcoholic petroleum ether. A plunger operated by a windshield wiper then ground the retinas at a speed of about 75 strokes per minute for 15 minutes. The extract was next decanted into a large test tube and pumped dry by a water aspirator with the aid of an ebullator tube to eliminate bumping. The dry extract was finally redissolved in 1.5 ml. of chloroform for spectrophotometric examination by means of a potentiometric photoelectric spectrophotometer (Bliss, 1946).

In order to distinguish the lipids released by red light from inert impurities, control extractions were carried out without illumination. The absorption spectrum of such extracts differed little from that of the pure solvent. An additional problem was the presence in the chick retina of even more rhodopsin than iodopsin. This required demonstration that rhodopsin did not bleach significantly in the red light used to bleach iodopsin. Digitonin extracts of powder bleached as described above showed that red light was without effect on its rhodopsin content.

#### *Lipids from Chick Retinas*

As may be seen in Fig. 1 the lipids from both iodopsin and rhodopsin showed the peak at about 390 m $\mu$  characteristic of retinene. The Carr-Price test was attempted but was unsuccessful due to the formation of turbidity on addition of the antimony trichloride reagent. Drying at 50°C. under vacuum for 5 minutes provided no improvement.

While the position of the peak at 390 m $\mu$  agreed with the published spectra of retinene (Krause and Sidwell, 1938), the absorption at longer wavelengths

was far too high. In particular there appeared to be a hump about  $470 \text{ m}\mu$ , a region where previously published spectra of retinene show practically no absorption. The trend of successive curves was strongly indicative of a labile substance with a maximum density about  $470 \text{ m}\mu$ .

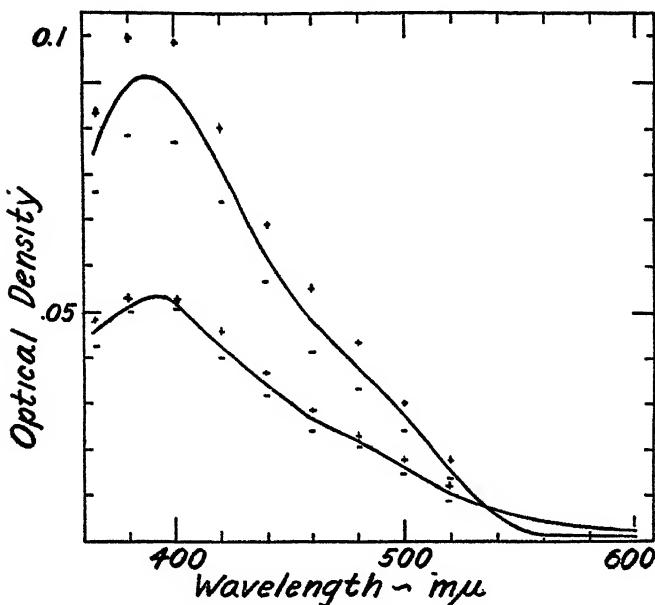


FIG. 1. Spectra of lipids from chick retinas, dissolved in 1.5 ml. of chloroform and measured in a 1 cm. cell. The curves are drawn through the average of duplicate experiments. Upper curve, effect of white light. Lower curve, effect of red light.

#### *Lipids from Frog Retinas*

In order to determine whether these anomalies represented a species difference or were due to the methods used, *Rana pipiens* powder was prepared and used in the same manner as the chick powder. It was found that 2 per cent alcoholic petroleum ether released retinene even in the dark. On reducing the alcohol to  $\frac{1}{2}$  per cent retinene was released only by bleaching by light and showed even more strikingly the peculiar lability mentioned above. The results of this experiment are shown in Fig. 2. The extrapolated curve for zero time of solution in chloroform is strikingly suggestive of the "transient orange" stage of rhodopsin solutions bleached in the cold (Lythgoe and Quilliam, 1938) and the "second dark component" noted by Wald (1938) in rapid, automatically recorded spectra obtained after bleaching at room temperature.

The height of the absorption spectra in the vicinity of  $470 \text{ m}\mu$  varied widely,

with Fig. 2 presenting in one experiment the different types of retinene spectra found in the course of many extractions. The usual spectra of frog retinene resembled those of chick retinene, as shown in Fig. 1. In all cases the curves passed through the sequence of Fig. 2, ending with the relatively stable spectrum of typical retinene.

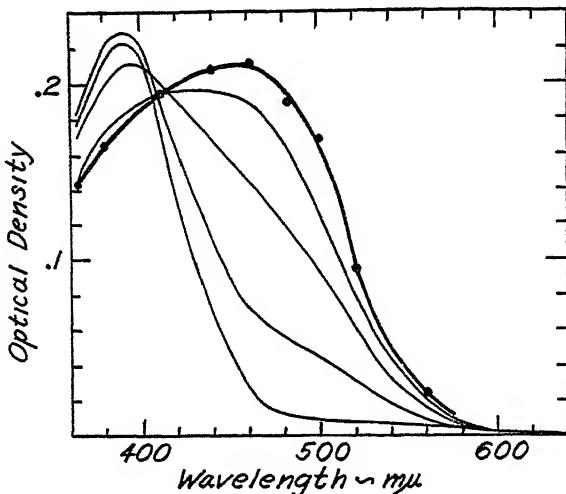


FIG. 2. Spectra of lipids from 14 frog retinas, dissolved in 1.5 ml. of chloroform and measured in a 1 cm. cell. The curves are drawn through experimental points at 20  $m\mu$  intervals at the following times: 0 minutes in chloroform-extrapolation, indicated by heavy line; 2 to 14 minutes; 15 to 28 minutes; 52 to 65 minutes; 192 to 207 minutes. Chloroform was added prior to the third and last series to replace loss due to evaporation. All curves were determined in order of increasing wavelength.

Because of the lability of the long wavelength component of the spectra, an attempt was made to minimize its breakdown by decreasing the temperature and time of bleaching and extraction. However, 10 minute extractions at 5°C., with a bleaching time of 1 minute did not differ significantly from 30 minute extractions at 27°C., with bleaching for 10 minutes.

#### DISCUSSION

It has become increasingly clear that the bleaching of rhodopsin is a complex chain of "light" and "dark" reactions. Some of the reactants in this chain have already been identified by Wald and incorporated into his well known cycle involving rhodopsin, retinene, and vitamin A. This cycle is certainly incomplete since it does not include Lythgoe's transient orange and indicator yellow.

Krause (1946) describes a complex lipid, provisual red, which he considers equivalent to transient orange. Provisual red shows absorption peaks at 330 and 440 m $\mu$  in absolute alcohol.

Indicator yellow is differentiated by Krause (1941) from visual yellow, which he believes to correspond to retinene, and which does not show the pH dependence of indicator yellow (Chase, 1936; Lythgoe, 1937). The lability of the retinene spectra found in the present experiments may well be due to the involvement of these poorly understood intermediates.

In any case the similarity of the bleaching products of both rhodopsin and iodopsin, extending even to their complex lability, testifies to the parallelism of rod and cone processes emphasized by Hecht (1937).

#### SUMMARY

A method is described for the preservation of iodopsin, the labile photopigment of daylight vision, by freeze drying *in vacuo*.

The lipids released by the action of light on rhodopsin and iodopsin are found to be similar and to possess a labile absorption spectrum in chloroform, with a rising peak at about 390 m $\mu$  and a declining peak in the region of 470 m $\mu$ . After the change is complete the absorption spectrum resembles closely that of retinene.

I am grateful for the encouragement of Professor Selig Hecht, in whose laboratory the exploratory experiments of this study were performed.

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# POTASSIUM ACCUMULATION IN THE PROXIMAL CONVOLUTED TUBULES OF THE FROG'S KIDNEY

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When the isolated kidney of the frog is soaked in solutions of varying ionic content, some ions are accumulated and others are lost by the tissue. It has been shown earlier (1) that similar changes occur with isolated muscle, the equilibrium conditions reached conforming with the predictions based on the assumption that the fibres are permeable to small anions and to potassium, but not to sodium. Such a permeability has been referred to as the standard permeability for ions (2) and applies to a wide variety of cells, though many exceptions may be instanced, in particular the special cells of the uriniferous tubules, or of the gills of fishes, which actively absorb or excrete Na ions.

In this connection impermeability of a cell to Na ions may be interpreted either as a complete blockage or a very slow entrance, the latter associated with a similar very slow exit requiring some energy expenditure. From considerations of the available energy and the entrance rate of KCl into the frog's sartorius (1, 3) an associated entrance of NaCl would appear to be feasible only at about one-hundredth to one-thousandth of the rate of KCl entrance, and it may not occur appreciably fast when compared with the life of the fibres. Throughout some hours at room temperature or 24 hours in the cold impermeability to Na ions apart from whatever views we may entertain as to Na extrusion may be interpreted for frog muscle as a real or potential blockage.

It is of interest to note that the entrance of small anions, such as chloride, along with potassium has been shown by Wilde (4) for the muscle of live rats, and although he obtained a product of the K and Cl concentrations outside the fibre which was only 50 to 60 per cent of the product within, yet if his figures be corrected for the fact that inulin (used to measure the interspaces) does not enter the red corpuscles in the capillaries whereas chloride does, the products approach each other. In other words the Donnan relation is at least approximately obeyed.

As shown here for the isolated kidney the greater fraction of the cells behave in a manner similar to muscle fibres, and can accumulate potassium against a gradient, but other cells act differently and actively extrude sodium and chloride when the kidney tissue is immersed in Ringer's solution. This loss of sodium and chloride is accompanied by a diminution of volume of the cells concerned. Such decrease in volume and loss of sodium are prevented by cyanide.

In order to understand the equilibrium between the K-accumulating cells of the kidney—appearing to act in this way like muscle—and the Ringer solution, it is necessary to measure (*a*) the proportion of the kidney tissue which is intercellular space, (*b*) the region that is freely permeable to Na in a steady state after soaking in Ringer fluid. Of the two it will appear that this latter is the more important “space” determination in studying the K accumulation in the normal tissue as the difference of the water therein from the whole tissue water measures the volume of fluid in the K cells.

The two regions of the nephron are identified as the proximal and distal tubules. The cells of the proximal tubules are shown to have an ion permeability very similar to that of the sartorius muscle, being impermeable to Na but letting through K and Cl. The cells of the distal tubule are permeable both to Na and the smaller K ion (to the latter at least from the blood side) and because of this K cannot be appreciably accumulated therein. The water of such cells can be regarded as so much free interspace with concentrations the same as the external solution, when calculating concentrations in the proximal tubules. (If in fact the Na and K concentrations in the water of the distal tubule tissue are not quite the same as outside, the size of such tissue in the kidney after immersion is relatively so small that little error arises here from assuming that they are so.)

With such a preliminary statement of similarities and differences between muscle and renal tissue regarding K and Na ions, the authors would like to state that a previous reading of the paper on K accumulation in muscle (2) would appear advisable for an easy understanding of the theoretical section.

### *Methods*

Kidneys from *Rana temporaria* were used throughout and treated as described for the sartorius muscle (2). Most of the experiments carried out refer to soakings in the cold (2–3°C.) in the modified Ringer solutions, and over a period of 24 hours. Others were carried out at room temperature, the kidneys being stirred by bubbling with a gas mixture for periods up to 3 hours.

### *Immersion Fluids*

*Ringer-Barkan Fluid.*—For the long period immersions in the cold, the solution contained in small conical flasks (50 ml. volume each with 4 to 5 kidneys) was first prepared by bubbling the gas mixture (3 per cent CO<sub>2</sub> and 97 per cent O<sub>2</sub>) through for 30 minutes, and transferring the well stoppered flasks to the refrigerator for 1 hour. The kidneys were then quickly immersed and the stoppered flasks replaced in the refrigerator.

The Barkan modification of the Ringer fluid (5) was chosen for reasons previously given (2) and it had the following standard composition:

NaHCO <sub>3</sub> .....	11.9	mm per litre
Na <sub>2</sub> HPO <sub>4</sub> .....	0.67	" "
NaH <sub>2</sub> PO <sub>4</sub> .....	0.10	" "

$\text{CaCl}_2$ .....	1.8 mm per litre
Glucose.....	3.9 " "

a pH of 7.3 being reached by bubbling with the gas mixture (3 per cent  $\text{CO}_2$ , 97 per cent  $\text{O}_2$ ). (The symbol mm is used throughout for millimols.)

To this was added the different  $\text{NaCl}$  and  $\text{KCl}$  quantities as required.

*Plasma-Salt Solution.*—Another fluid occasionally used simulated closely the inorganic composition of frog plasma. It is described in a previous communication (2).

*Hemoglobin-Ringer Fluid.*—The hemoglobin was prepared by dialysing heparinized rabbit blood, previously diluted 1 in 4, for several hours until practically free of the  $\text{Cl}^-$  ion and then making up with  $\text{NaCl}$  (to 0.65 per cent strength) and the usual Ringer ingredients (but without bicarbonate).  $\text{KCl}$  was also incorporated to the extent of 30 m.eq. per litre to prevent  $\text{K}^+$  losses and maintain the cell membranes in an apparently better condition, as to permeability, than without such addition.

*Inulin-Ringer Fluid.*—This was similar to the previous fluid but contained 1.5 per cent inulin instead of hemoglobin.

#### *Analyses.*—

Sodium and potassium were determined as previously described (1).

Chloride was determined by a microdiffusion method (6, 7).

*Hemoglobin.* Single kidneys were immersed—over 24 hours in the cold—in rather wide stoppered tubes containing a few milliliters of hemoglobin-Ringer fluid, then removed, surfaces quickly dried and transferred to 1 ml. hemoglobin-free Ringer solution in similar tubes for another 24 hours; after which 0.8 ml. was pipetted off into small centrifuge tubes, 0.2 ml. strong alkali added, mixed, and the contents warmed for about 5 minutes in the water bath, centrifuged, and the clear fluid examined spectrophotometrically. The original hemoglobin-Ringer fluid diluted 1/100 was used as a standard, and the blank values determined by immersion of the companion tissues in a similar way, hemoglobin being omitted.

*Inulin.* For experiments with single kidneys, immersions and diffusions were carried out as for the hemoglobin, 1.5 ml. of a 1.3 per cent  $\text{Na}_2\text{SO}_4$  solution being used for the second 24 hours' soaking. For the inulin analyses, duplicate 0.5 ml. samples were used. These were transferred to the outer chambers of Conway units. Into the inner chamber of each unit was pipetted 1.3 ml. of N/50  $\text{Ba}(\text{OH})_2$  containing 5 per cent of B.D.H. universal indicator and the lid (with fixative) placed in position. Into the outer chamber was then run 2 ml. of an oxidation mixture consisting of 40 per cent  $\text{H}_2\text{SO}_4$  saturated with permanganate. (This mixture had been left exposed for some hours in a large beaker to allow the escape of  $\text{CO}_2$  from traces of carbonate as impurity in the permanganate, and the mixture was made up in this way each day.) After 2 hours in unit, 1 ml. of the  $\text{Ba}(\text{OH})_2$  solution was removed from the central chamber into a small tube and titrated to a green colour with N/25 HCl from a Conway burette (7).

## RESULTS

### *I. Experiments to Investigate the Volume of the Intercellular Spaces in the Isolated and Immersed Kidney*

(a) *The Hemoglobin Ratio.*—This was investigated for thirty-five single kidneys soaked over 24 hours in the cold in the hemoglobin-Ringer fluid, the

hemoglobin diffused into the kidney being re-diffused out into Ringer fluid over another 24 hours. The mean value of the ratio:—

$$\frac{\text{Hemoglobin per kg. original weight}}{\text{Hemoglobin per kg. external fluid}}$$

was  $0.175 \pm 0.004$  (giving standard deviation of mean). For similar experiments with N/250 cyanide in the hemoglobin-Ringer solution the mean for twenty-one kidneys was  $0.174 \pm 0.005$ .

TABLE I  
*Mean Ratio for the Immersed Kidneys*  
(Ratio =  $\frac{\text{gm. per kilo original weight}}{\text{gm. per kilo external fluid}}$ )

		Active tissues		Inactive tissues	
		Ratios	Weights after/before	Ratios	Weights after/before
Hemo-globin	Single kidneys in the cold	$0.175 \pm 0.004$ (35)	0.81	$0.174 \pm 0.005$ (21)	0.97
	" "	$0.23 \pm 0.012$ (28)	0.85	$0.27 \pm 0.016$ (10)	1.05
Inulin	Sets of 4 kidneys, room temperature	$0.23 \pm 0.02$ (10)	0.82	$0.32 \pm 0.028$ (6)	0.96
	" "	$0.26 \pm 0.014$ (26)	0.82	$0.55 \pm 0.023$ (26)	1.10 (for external K values less than 100 mm per kg.)
Na					

The  $\pm$  addition gives the standard deviation of mean. The figures in parentheses give the number of experiments.

Concerning the adequacy of the soaking period of 24 hours, experiments with a 48 hour soaking period with subsequent 48 hour diffusion proved rather unsatisfactory owing to the development of some turbidity indicating tissue breakdown. The following experiment was also performed. Twelve kidneys first soaked for 24 hours in the cold were transferred to Ringer solution (about 25 times their volume) at room temperature and stirred gently by a mechanical stirrer. After 2 hours the diffusion had come to an end as evidenced by the fact that the ratio calculated after 30 minutes' stirring and examination of a sample of the fluid was 0.11 with ratios of 0.167 and 0.168 after 120 and 180 minutes. It is true that only the 24 hour entrance of hemoglobin in the cold

would emerge in these experiments, but the rapidity with which it comes out points to the adequacy of the soaking period, and such conclusions are in turn supported by the subsequent inulin investigations.

(b) *The Inulin Ratio.*—The inulin ratio was investigated in a similar manner to that of hemoglobin with single kidneys in the cold, but experiments with groups of kidneys were also made at room temperature. The mean value of the ratio for twenty-eight experiments is  $0.23 \pm 0.01$  (Table I). With cyanide the figure increases slightly but scarcely significantly to  $0.27 \pm 0.02$ . At room temperature on the other hand (groups of four kidneys, bubbling for 2 hours in inulin-Ringer fluid) the mean change is from 0.23 to 0.32.

(The adequacy of the diffusion times was shown in the experiments at room temperature. After 60, 90, and 120 minutes the ratios were 0.214, 0.232, and 0.235, which give the means for three sets of seven groups of four kidneys.)

If instead of the inulin ratio as given above the relation be expressed as a "permeation" previously defined (8) and which may be written

$$100 \times \frac{\text{gm. per kg. water after immersion}}{\text{gm. per kg. external fluid}}$$

then the value for inulin in the cold is 32 and 36 at room temperature which may be compared with 41 for sections of rabbit kidney at  $37^{\circ}\text{C}$ . With cyanide at room temperature the value for the frog's kidney rises to 41 and for the cyanide-perfused rabbit kidney it was found to be 56.

## *II. Experiments to Investigate the Proportion of the Isolated Kidney Showing Free Entrance of NaCl*

The measurement of this region is essential for determining the K-accumulating space, for wherever Na with accompanying Cl or  $\text{HCO}_3^-$  ions enter freely, K cannot be accumulated, in the manner described for muscle (2).

It will be seen later that for the theoretical treatment it is the most important "space" determination. In volume it may be expected to be at least as great as the space indicated by the inulin ratio, and in fact for the isolated immersed kidney the Na ratio reaches a value but little different from that of inulin, but certain peculiarities such as a marked reduction in renal volume on immersion associated with similar reduction in the Na ratio require special consideration.

(a)  *$Na$  and Cl Ratios for the Fresh Kidney with Respect to the Plasma Values.*—The mean Na content of the fresh kidney is  $41.2 \pm 0.6$  mm per kilogram (seventy-nine analyses of groups of four kidneys, Table II) and the mean Na content of plasma as shown in Table II is 103.8 mm per kg. This would mean a Na ratio of 0.397, or 0.38 with the external concentration referred to plasma water. For reasons considered later, 0.39 may be taken as close to the true mean value. Similarly the mean Cl content of the kidney is  $30.1 \pm 0.8$  (twenty

analyses) and that of the plasma is 74.3, thus giving a ratio of 0.405. The Na and Cl ratios for the frog's kidney with respect to plasma are thus very

TABLE II  
Normal Mean Values for Frog Kidney (*Rana temporaria*)

Substance	Kidney	No. of analyses	Plasma
	mm/kg.		mm/kg.
K.....	60.0 ± 0.6	67	2.5 (F)
Na.....	41.2 ± 0.6	79	103.8 (F)
Cl.....	30.1 ± 0.8	20	74.3 (F)
HCO <sub>3</sub> .....	—	—	25.4 (F)
Water.....	816 gm./kg.	5	954 gm./kg. (S)

Each kidney analysis was carried out on four kidneys from four frogs. (F) represents Fenn's results (19) for plasma and (S) the figure of Schultz *et al.* as quoted by Fenn (19).

The ± values give the standard deviation of the mean.

TABLE III  
Mean Values for Series of Kidneys Immersed for 24 Hours at 2-3°C. in Ringer Solutions with Fixed Na but Different K Concentrations

External concentrations			Renal concentrations			Weights after/before	Dried weights gm./kg.
K	Cl	Total	mm/kg.	Na	Cl		
mm/litre	mm/litre	mm/litre	mm/kg.	mm/kg.	mm/kg.		
6	82	192	50.8 (4)	21.5 (4)	26.3 (2)	0.863 (12)	160 (2)
12	88	204	57.1 (2)	20.9 (3)	—	0.896 (4)	—
18	94	216	62.3 (4)	19.4 (3)	35.3 (2)	0.848 (9)	164 (2)
30	106	240	71.1 (8)	22.9 (4)	38.5 (2)	0.852 (13)	163 (2)
60	136	300	88.0 (2)	26.5 (2)	54.8 (2)	0.831 (8)	165 (2)
90	166	360	111.0 (5)	24.1 (4)	70.6 (2)	0.828 (13)	169 (2)
120	196	420	123.4 (2)	25.1 (2)	88.0 (2)	0.811 (8)	157 (2)
150	226	480	144.0 (5)	24.1 (4)	108.0 (2)	0.799 (13)	154 (2)
180	256	540	167.8 (2)	31.7 (2)	122 (2)	0.812 (8)	170 (2)
210	286	600	184.2 (4)	39.5 (4)	143 (2)	0.815 (11)	173 (2)

All concentrations in the kidney are given as mm per kilogram of the weight before immersion. Bracketed figures give the number of experiments. There was constant Na concentration of 86 mm/litre in the immersion solutions. Total external diffusible anion is found by adding 13 to chloride values.

similar, and may be compared with those for muscle, namely 0.22 for Na, and 0.14 for Cl, which show considerable relative difference.

(b) *The Change in the Na Ratio for the Immersed Kidney and the Corresponding Volume Change.*—When the kidneys are immersed for 2 hours at room temperature or for 24 hours in the cold (2-3°C.) the Na ratio falls and also the volume. As will appear from the data in Table III, (referred to again below), when the external concentration of the Ringer-Barkan fluid is 240 mm per litre

and the external K value 30 mm (Na concentration = 86 mm per litre) the ratio has fallen from about 0.40 to  $22.0/86 = 0.266$  and the volume from 100 to 85 per cent. (Similar values are observed throughout the table with constant Na and different external K values, except where at the highest K concentrations, there is a relative increase in the Na ratio.) In the presence of N/250 cyanide no fall of Na ratio occurs, but rather an increase (as may be seen from data of Table IV), and provided the external potassium is not very high the volume of the kidney also increases. Volume changes over 2 hours for kidneys immersed in ordinary Ringer-Locke solution (0.7 per cent NaCl) at room temperature are shown in Fig. 1 (a limiting value being nearly reached within the period).

TABLE IV  
*Values as in Table III with the Immersion Fluids Containing N/250 Cyanide*

External concentrations			Renal concentrations			Weights after/before	Dried weights gm. per kg. of original tissue
K mm/litre	Cl mm/litre	Total (c) mm/litre	K mm/kg.	Na mm/kg.	Cl mm/kg.		
6	82	192	35.5 (1)	70.2 (1)	41.8 (2)	1.17 (4)	183 (2)
12	88	204	35.0 (1)	—	—	1.20 (1)	—
18	94	216	46.3 (2)	43.3 (2)	49.0 (2)	1.14 (4)	187 (2)
30	106	240	64.6 (2)	57.4 (2)	52.9 (2)	1.06 (6)	176 (2)
60	136	300	80.6 (1)	49.2 (3)	65.3 (2)	1.02 (5)	187 (2)
90	166	360	97.8 (1)	56.6 (2)	85.3 (2)	0.98 (6)	184 (2)
120	196	420	130 (1)	68.4 (1)	98.0 (2)	0.92 (4)	184 (2)
150	226	480	152 (1)	53.7 (3)	116.0 (2)	0.91 (6)	189 (2)
180	256	540	—	56.3 (3)	146.0 (2)	0.92 (5)	184 (2)
210	286	600	184 (1)	61.7 (2)	165.0 (2)	0.94 (5)	202 (2)

All renal concentrations given as mm per kg. of weights before immersion. The immersion fluids contained a fixed Na concentration of 86 mm per litre.

From these experiments it will be seen that the original Na ratio in the kidney is about double that for hemoglobin or inulin ratios for the immersed kidneys, but that after immersion the value falls to near that of inulin and there is a corresponding loss of water.

The total average for the Na ratio after 24 hour immersions is 0.26, the average inulin ratio being 0.23.

(c) *Relative Diffusion Rates of Na, Cl, and K from the Isolated Kidney into Isotonic Glucose.*—On considering the high Na ratio of 0.39 for the fresh kidney and the comparative effect of cyanide, with the isolated kidney it would appear that Na is present not only in the intercellular spaces but a proportion is also intracellular. The question arises as to whether this intracellular Na is uniformly distributed throughout the renal cells or is restricted to some region of the nephron. The diffusion into isotonic glucose was designed to provide

data relevant to this question; for if Na is contained in appreciable amounts in cells which contain K, then since it is the larger ion, it could be expected to emerge relatively more slowly than K when immersions are made in K-free isotonic glucose. If, on the other hand almost all the Na emerges very rapidly while only a fraction of the K diffuses out, it would appear as the simplest explanation that the intracellular Na is confined to a separate region. Sartorius muscles were used for comparison.

These experiments were carried out with twenty kidneys (or sartorius muscles) immersed at room temperature in 3.8 per cent glucose and stirred

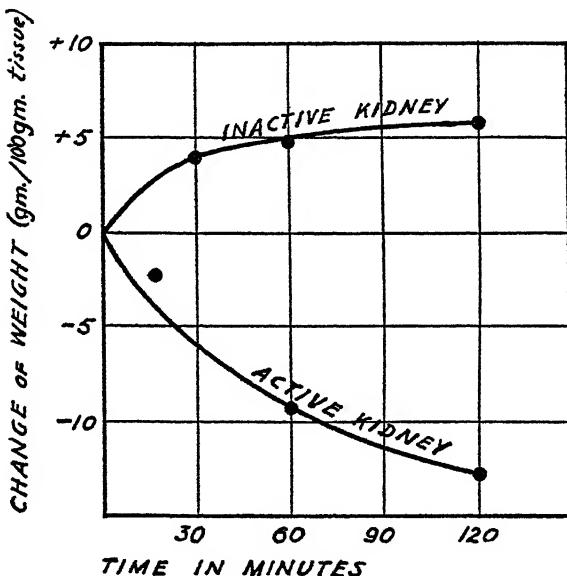


FIG. 1. The lower curve gives weight changes at room temperature for five kidneys from five frogs immersed in Ringer-Locke solution containing 0.7 per cent NaCl; the upper curve for the companion tissues in a similar solution containing N/500 KCN.

by bubbling with oxygen, samples being taken at intervals for Na and K analyses, the tissues at the end being also analysed. For the chloride analyses groups of four kidneys were taken from a bath of twenty immersed kidneys as before, and analysed for chloride. Companion tissues were taken for the determination of the original chloride.

Fig. 2 illustrates the relative rates of emergence of Na, Cl, and K. It will be seen that nearly all the Na and Cl have left the kidney and diffuse out at practically the same rates, while only 10 per cent of the renal K is lost. After 60 minutes only 2 to 3 mm of the original 41 mm per kg. of Na are left. Comparing these results with those for the sartorius muscle it will be seen that about

40 per cent of the Na therein—approximately equivalent to the amount held in the fibres—is lost very slowly, while the free Na and Cl in the extracellular spaces are lost rapidly.

### *III. Experiments at 2–3°C. Showing KCl Accumulation, with Constant Na Concentration in the Immersion Fluid*

For the sartorius muscle it was shown that large amounts of K as KCl could be accumulated with but little volume change if immersions were made in Ringer solutions in which the Na was held constant throughout the series and the KCl much increased. For the muscle series the constant [Na] in the Ringer fluid was 86 mm per litre being somewhat lower than the normal 104 mm per litre plasma. A small volume increase was therefore shown throughout, but largely independent of the K content (there being in fact a slight

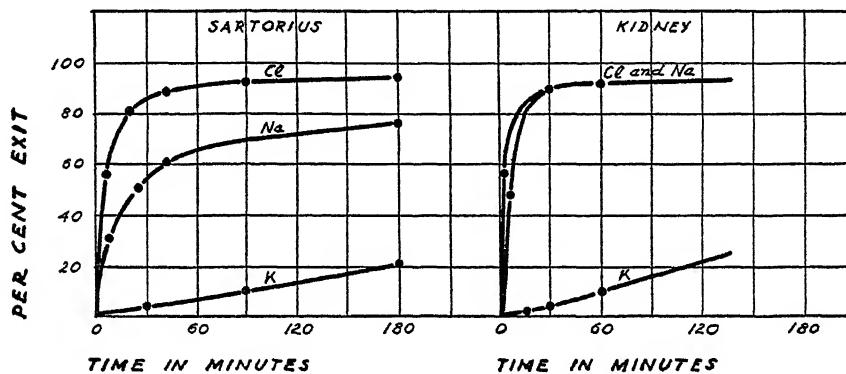


FIG. 2. Diffusion of Na, K, and Cl from batches of twenty kidneys and sartorius muscles (from ten frogs) immersed in 3.8 per cent glucose.

relative fall in weight as the external KCl was increased towards 300 mm per litre). Similar solutions were used for comparative purposes for the renal immersion, and the results are shown in Table III. The external solution had for these experiments the following composition (mm per litre) apart from the KCl increase:—

Na.....	85.9	HCO <sub>3</sub> .....	11.9
Ca.....	1.8	Cl.....	76.2
Glucose.....	3.9	P (as phosphate mixture).....	1.8

and the immersions were made as described under Methods.

The results are shown in Table III. The results for immersion in similar solutions containing N/250 sodium cyanide are shown in Table IV. It will be seen that K is largely accumulated both in the active and inactive kidneys, and to somewhat the same amounts. The Na changes are very different, there

being a mean loss of 18 mm per kg. with the active kidneys up to an external K of 180 mm per litre the results at each K level differing from this no more than 4 mm per kg. The corresponding mean weight loss is 16 per cent. On the other hand the cyanide kidneys show increase of Na throughout, the mean increase being approximately 16 mm per kg. Increases in volume are likewise shown when the external K is not very high.

TABLE V  
(*Immersions at 2–3°C. in Isotonic Mixtures with Varying K Concentrations*)

$k$ (Potassium concentration in external fluid)	[Na (Sodium concentration in external fluid)]	$\frac{100}{[Na] + 5.7}$	Mean weight of 1 kg. of fresh kidney after immersion	Ranges and number of observations (each observation = weight of 4 kidneys)	$k_2$ (Potassium concentration of immersed kidneys per kg. fresh weight)
mm/litre	mm/litre				
10	107	0.89	0.81	0.75–0.83; 6	
20	97	0.97	0.80	0.76–0.83; 5	
30	87	1.08	0.86	0.82–0.93; 9	69 (2 observations)
40	77	1.21	0.91	0.84–0.93; 6	
50	67	1.38	0.99	0.97–1.03; 6	
60	57	1.59	1.06	1.02–1.11; 6	
70	47	1.89	1.18	1.05–1.25; 6	145 (2 observations)
80	37	2.34	1.34	1.26–1.46; 9	
90	27	3.06	1.64	1.46–1.87; 14	126 (2 observations)
100	17	4.40	2.00	1.89–2.08; 6	

#### IV. Experiments Showing K Accumulation in Isotonic Solutions

When muscle is immersed in Ringer solution in which K is substituted for Na in equivalent relation, K is accumulated with large volume changes, following a theoretical relation up to a weight increase of about 70 per cent, or to about 100 per cent increase of the fibre water. Immersion in similar solutions was carried out with frogs' kidneys. The solutions had the following composition:—

Total Na and K.....	117 mm per litre
Cl.....	107 mm per litre
HCO <sub>3</sub> .....	11.9 mm per litre
Ca.....	1.8 mm per litre
P (as phosphate mixture).....	0.8 mm per litre
Phosphate.....	3.9 mm per litre
Total mm per litre.....	243 mm per litre

These were also equilibrated with the gas mixture as before. The results for 24 hour immersions are shown in Table V, and it will be seen that a great relative increase of renal volume occurs as the Na content falls outside. The

mean weights range from 0.81 (of the original weight) with a K value of 10 mm per litre outside to 2.00 with 100 mm per litre external K. In Table VI it is shown that no further appreciable volume change occurs after a second 24 hour immersion.

TABLE VI

(Immersions at 2-3°C. in isotonic mixtures as in Table V; comparison between weights referred to 1 kg. fresh weight after 24 and 48 hours for one set of forty kidneys—four in each mixture.)

<i>k</i>	Weights after 24 hrs.	Weights after 48 hrs.
10	0.83	0.83
20	0.82	0.83
30	0.84	0.84
40	0.92	0.91
50	1.01	1.00
60	1.03	1.02
70	1.22	1.21
80	1.38	1.36
90	1.64	1.71
100	2.07	2.08

TABLE VII

(Immersions at 2-3°C. isotonic mixtures as in Table V, but containing N/500 cyanide.)

<i>k</i>	100 [Na] + 5.7	Mean weights of immersed kidneys referred to 1 kg. fresh weight		
		After 24 hrs.	After 48 hrs.	After 72 hrs.
10	0.87	0.97 (4)	1.05 (3)	1.11 (2)
20	0.95	0.98 (3)	1.02 (3)	1.09 (2)
30	1.06	1.05 (4)	1.13 (3)	1.18 (2)
40	1.18	1.09 (3)	1.17 (3)	1.19 (2)
50	1.34	1.17 (4)	1.25 (3)	1.28 (2)
60	1.54	1.23 (3)	1.33 (3)	1.42 (2)
70	1.83	1.31 (4)	1.44 (3)	1.50 (2)
80	2.23	1.46 (3)	1.58 (3)	1.65 (2)
90	2.88	1.53 (4)	1.70 (3)	1.79 (2)
100	4.03	1.54 (3)	1.65 (3)	1.78 (2)

Experiments were also carried out in which volume changes in similar isotonic solutions but containing M/500 or M/250 cyanide were investigated. Only the results with M/500 cyanide are given (Table VII) since those with M/250 cyanide did not appreciably differ therefrom. The results in Table VII show that the increase in volume is not ended after 24 hours but continues up to at least 72 hours.

In Fig. 3 the kidney weights as ratios of the fresh weights are plotted against the reciprocals of ([Na] + 5.7) (the figure 5.7 corresponding to the small con-

centration of Ca and glucose). The upper line gives the results after 24 hours' immersion in cyanide-Ringer fluids and the lower the results without cyanide. It will be seen that the effect of cyanide is to raise the level of the line, but it has no influence on its slope. One rather interesting difference is the manner in which with cyanide the linear relation is somewhat abruptly departed from at a lower level of volume increase, showing that the differential permeability of the cells involved is then not so resistant to distension of the membrane. It

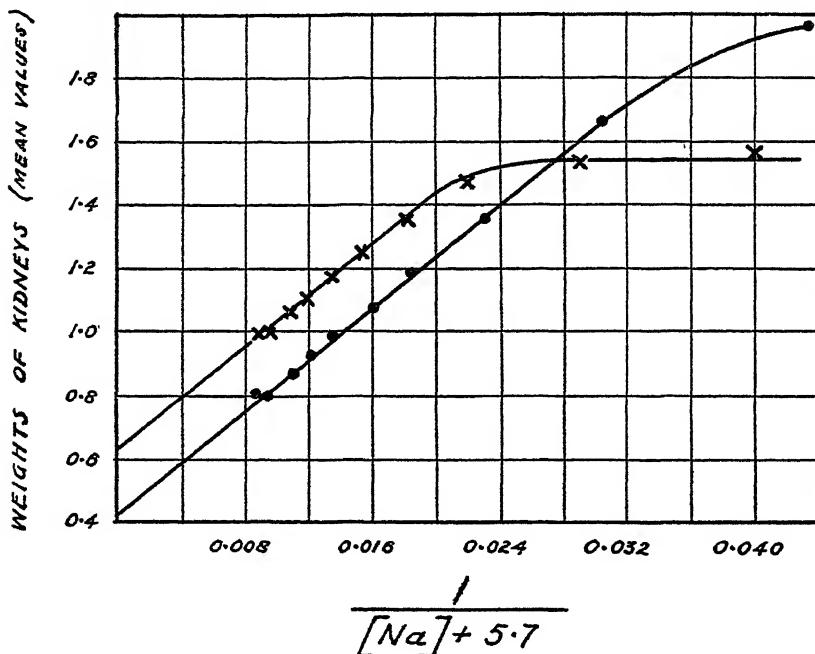


FIG. 3. Mean weight of kidney after 24 hours' immersion at 2-3°C. in isotonic Ringer fluids, with varying Na and K concentrations, plotted against the reciprocal of  $([Na] + 5.7)$ , [Na] being the external sodium concentration (mm per litre) and 5.7 the sum of the Ca and glucose concentrations.

The upper curve gives the results in the presence of  $n/500$  sodium cyanide.

is also of interest that though the renal volume increases with cyanide after 24 hours the slope of the line above considered remains unchanged (as would appear from plotting the results in Table VII).

#### THEORETICAL SECTION

##### I. K and Na Regions of the Nephron

When the frog's kidney is immersed in Ringer solution, as shown above the Na ratio falls from an initial value of 0.39 to 0.26, which is near the inulin

figure of 0.23. From the high Na ratio of the fresh kidney, it is obvious that much of the Na is initially contained in cells of the nephrons, and is actively extruded. This is shown by the cyanide effect, when the Na ratio no longer falls but rises somewhat, with a corresponding increase in weight (provided the external K is not very high). The question then arises, *is the Na present in all the cells of the nephron and actively extruded therefrom or is it present only in a fraction.* It would appear that this can be answered effectively in the following way. If the Na is not present in the major fraction of the nephron and exists only in a special region the volume of which is indicated by the Na ratio, then the major fraction may be taken as impermeable to the Na ion, and if so it should act—at least within a certain range—practically as an osmometer for the Na ion concentration (even in the presence of KCl, with the membrane freely permeable to K and Cl ions).

Examining then how this relation is fulfilled, if we take the weight of the immersed kidney as  $W_i$ , and subtract from this the Na ratio of 0.26 plus 0.16, (the latter being the mean weight of the solid material), we obtain  $W_i - 0.42$ . This may be expected to be proportional to the reciprocal of the Na ion concentration. We must take into account however, that a small amount of Ca is present in the fluid used, and also a little glucose, both adding to 5.7 mm per litre. By analogy with muscle the membranes of these renal cells may be expected to be impermeable to these as well as to the Na ion. Taking then the data of Table V where the total of the Na and K ion concentration remains the same (117 mm per litre, with total mm of 243 in the solution) but is relatively varied, and plotting ( $W_i - 0.42$ ) against the reciprocal of the concentration ([Na] + 5.7) we get, up to an external K of 90 mm per litre a linear relation, the line of best fit passing through or very close to the origin. This will appear evident from Fig. 3 (lower curve) in which the line through the  $W_i$  values cuts the ordinate at 0.42. Attention may here be directed to the fact that the weight change of ( $W_i - 0.42$ ) is from 0.34 to 1.18, a very large relative increase for cell water. The existence of this relation would seem explicable only by the effective impermeability to the Na ion of the greater fraction of the nephron, the remainder plus intercellular spaces being freely permeable to Na.

As supporting evidence we have the fact that Na and Cl diffuse together and almost completely from the immersed kidney within about 30 minutes after immersion in isotonic glucose as shown in Fig. 2. Within the same period the kidney loses only a small fraction of its potassium. This slow loss of K is like that from the muscle fibre when immersed in K-free solutions.

Can the conclusion be avoided that the larger fraction of the nephron is impermeable to Na? It might perhaps be entertained that the permeability is unidirectional and though the Na ions cannot pass back, they could still enter from the lumen side. This is scarcely tenable when we consider the long period of immersion and the practical certainty that NaCl will diffuse

down through the glomeruli, and backwards up the lumen of the nephron as well as through the cellular region which is taken as permeable to the Na and Cl; and there is also the fact that, as seen from Fig. 3, up to high levels of distension *cyanide does not appreciably affect the nature of the volume change resulting from reduced Na concentration*, for the line of renal weight against the reciprocal of ( $[Na] + 5.7$ ) retains the same slope.

In this first theoretical section we shall consider it sufficient then to conclude that there exists a large region of the nephron which has a permeability like that of the muscle fibre, impermeable to Na but permeable to K and Cl which can be greatly accumulated in the isolated kidney, and on the other hand there exists a smaller region which is freely permeable to Na and actively extrudes it from the cells in the immersed kidney decreasing very markedly in volume. It will be shown then that the theory used to explain K accumulation in the muscle fibre can in turn be very successfully used to explain K accumulation in the K region of the nephron. Before considering this, the manner of calculating K and Cl values in the K region will be dealt with.

#### *Calculation of K and Cl in the Water of the K region*

*Fresh Kidneys.*—For these the Na ratio may be taken as giving the region of free Na entrance. From Table II the mean Na content per kilogram is  $0.412 \pm 0.006$  (seventy-nine analyses) and the mean plasma value is 103.8, from which the Na ratio is  $\frac{41.2}{103.8} = 0.397$  or 0.40 for 1 kg. (the Cl ratio is slightly higher, being 0.405). More strictly we should here consider the mm Na per kilogram of plasma water, which would mean dividing by 0.954 (Table II), but, at the same time we should need to multiply by a factor corresponding to the ratio of [Na] in extracellular water to that in plasma water. Since that is usually taken as 0.95 (though it may be expected to be nearer to unity in the frog) the two factors would largely cancel (or at least may be expected to do so within 1 or 2 per cent). A figure of 0.39 may be stated as probably very close to the true mean value. We have then for the mean value of [K] in 1 kg. of proximal tubule water ( $k_1$ ) of fresh kidneys:—

$$k_1 = \frac{k_0 - 0.39k_p}{1.00 - W_d - 0.39}$$

$$= \frac{60.0 - 1.0}{1.00 - 0.184 - 0.39} = 139 \quad (1)$$

$k_0$  being the mm K per kilogram of the fresh tissue,  $W_d$  the dry weight of 1 kg. of kidney and  $k_p$  the K concentration in plasma water.

If the chloride content of the proximal tubule water be calculated in the same way, the amount is so small, that an error in the determination of the region of free Na and Cl entrance has a relatively large effect. For more

accurate results the Donnan relation may be assumed. The product of the K and Cl concentrations in plasma water, from the data of Table II, is 204. Since the K concentration in the proximal tubule water is 139, the Cl concentration is therefore  $204/139 = 1.5$  mm per litre.

This chloride calculation may be qualified by such consideration as that the K in protein-free dialysates of dog's sera *in vivo* (9) shows a ratio with respect to the plasma only 86 per cent that expected from the Na ratio, or that for similar dialysates *in vitro* (10), the K ratio is 91 per cent that of the Na value (a summary of such results is given by Peters (11)); but such qualifications even if applying to the frog would have very little effect on the absolute figure for Cl in the cell water of the K accumulating region of the kidney.

In a similar way it may be calculated that the concentration of the  $\text{HCO}_3^-$  ion in the water of the proximal segment is 0.5 mm per kg.

*Immersed Kidneys.*—All concentrations giving direct analytical results, measurements of weights and of dry weights are referred for uniformity and convenience to 1 kg. of fresh weight of kidney.

The Na ratio of 0.26 was considered above for measuring the volume of the kidney freely permeable to the Na ion (and therefore, it may be assumed, to the K ion outside the K region). The line drawn through the kidney weights (as ratios of the fresh weights) minus 0.42 (0.26 plus the mean dry weight of 0.16) when plotted against the reciprocal of  $([\text{Na}] + 5.7)$  passes through the origin, or very close thereto, as may be judged from Fig. 3, and this makes it reasonably certain that the figure of 0.26 is close to the true value for the Na-permeable space. Its lower limit would in any case be the inulin ratio of 0.23 for wherever inulin goes it may be assumed that the Na ion would likewise go, and it would in fact make little difference to the results whether 0.23 or 0.26 figures were used for the space freely permeable to NaCl, but clearly the 0.26 figure is the better.

We may write then:—

$$(\text{Water of K region per kilogram fresh kidney}) = W_i - W_d - 0.26 \quad (2)$$

where  $W_i$  = the weight after immersion and  $W_d$  the corresponding dry weight, 0.26 being that of the water freely permeable to Na.

Into this Na region it may be assumed that the external K—as a smaller ion—will likewise have free entrance, but only to a concentration approximately equal to the external value. With a mean dry weight of 0.16 (as ratio of the fresh kidney weight) the mm per kilogram of water in the proximal tubule ( $k_1$ ) may be written:—

$$k_1 = \frac{k_0 - 0.26k}{W_i - 0.42} \quad (3)$$

where  $k_0$  is the mm K per kilogram of the fresh tissue.

The mm Cl per kilogram water in the proximal tubule may be similarly calculated.

## *II. The Mechanism of Potassium Accumulation in the K Region of the Nephron*

### *(a) Theory of the Potassium Equilibrium*

The theory of such K accumulation has been already treated in some detail for muscle (2) and it is only necessary here to state the essential equations.

It is a practical need in developing or using equations such as those for the volume of the cell water (Equation 7) to have a symbolism as compact as possible. The following symbols have been chosen, very similar to those used in dealing with muscle.

In "cell water" of the K-accumulating region

$k_1, l_1, h_1$  = mm K, Cl, and H ions per kg. of cell water.

$\Sigma d_1$  = mm diffusible anions per kg. of cell water.

$\eta$  = The "idiomolar" value, or total millimols of non-diffusible substance per kg. of the original cell water.

$\epsilon$  = The "electrostatic equivalent" of  $\eta$ , or the surplus of negative charges on the non-diffusible electrolytes, expressed as milli-equivalents per litre.

$V$  = Volume of water in the number of cells which for fresh tissue contain 1 litre or 1 kg. of cell water.

*For external solution:*

$k, l, h$  = mm K, Cl, and H ions per litre external solution.

$c$  = total external concentration, as mm per litre.

$\Sigma d$  = mm diffusible anions per litre of external solution.

From the previous considerations the cell water in the K-accumulating region of 1 kg. of fresh kidney is  $1.00 - 0.18 - 0.39 = 0.43$ , and for the immersed kidney it is  $W_i - 0.16 - 0.26$  or  $W_i - 0.42$ , so that  $V$  for the immersed tissue is  $\frac{W_i - 0.42}{0.43}$ .

Concerning the  $\eta$  value it seemed advisable to have a word which would indicate its nature, and the term "idiomolar value" is suggested as expressing the fact that the molecules referred to, are entirely retained within the cell itself. The  $\epsilon$  symbol is in turn termed the "electrostatic equivalent" of  $\eta$  since it determines the number of the K ions in excess of the small amount of diffusible anions, which are held electrostatically within the cell.

As in muscle the cell membrane in the K-accumulating cells of the kidney is considered to be permeable to cations and anions, but within certain size limits, so that whereas Na is excluded, K is allowed through; and though Cl passes the membrane, anions such as the esters of phosphoric acid do not. It was shown for muscle that much KCl could be accumulated without volume

change, the K ions moving against a concentration gradient to an equilibrium determined by the Donnan relation. At the same time there was no change in the Na value in the muscle which remained a constant and rather small fraction of that outside. This with the fact that cyanide does not affect the Na control of volume appears to be an exact proof of the relative Na impermeability in muscle, and it will be seen that similar conditions prevail for the cells of the K-accumulating region in the kidney.

*(b) Accumulation of Potassium in the K Region with Fixed External Na Concentration*

For immersed muscle with the mean  $\eta$  value of 105.5, very little different from  $\epsilon$  ( $= 103.5$ ) it was shown that the volume of the fibre water was proportional to the sodium ion concentration, and it was only necessary to hold this value constant to maintain the value of  $V$ . Here the  $\eta$  and  $\epsilon$  values differ appreciably and though  $V$  does not increase with the accumulation, it decreases somewhat, but in accordance with theoretical expectation.

The equation for the potassium concentration in the K-accumulating region (2) is:—

$$k_1 = c/2 - (\eta - \epsilon)/2V \quad (4)$$

When Na is held a constant, though  $V$  changes but little over the widest ranges of external K, and of K accumulation, it does change a little, and so also  $\eta$  and  $\epsilon$ , but the total value of  $(\eta - \epsilon)/2V$  is at all times small compared with  $c/2$ , consequently the slope of the line of  $k_1$  values against the total external concentration is approximately  $c/2$ .

Where the slope of the line of  $k_1$  values against " $c$ " cuts the ordinate at  $c = 0$  the value of  $(\eta - \epsilon)/2V = -28$  is obtained.

Equation 4 becomes therefore:—

$$k_1 = c/2 + 28 \quad (5)$$

The last two columns of Table VIII show the agreement between the experimental and theoretical values. From an external potassium value of 18 mm to 210 mm per litre, the divergence between the  $k_1$  values and that from Equation 5 is less throughout than 5 per cent.

*The  $\eta$  and  $\epsilon$  Values.*—It will be seen from Table VIII that the mean value of  $\eta$  is 76 for such immersed tissues, and varies from this with increasing accumulation no more than could be expected from the sampling error. The mean value of  $\epsilon$  is 136 but there is clearly an increase with increasing " $k$ ." This is to be expected theoretically, as discussed elsewhere for muscle (2) but with immersed muscle it is compensated by slight losses of non-diffusible material.

With regard to the  $\eta$  and  $\epsilon$  values for the fresh kidney, such may be calculated from the equations given under Table VIII where  $V = 1.00$ , " $c$ " (for plasma)

= 233 (2),  $k_1 = 139$ , and  $\Sigma d_1$ , comprised almost entirely of Cl and HCO<sub>3</sub>, has the value of  $1.5 + 0.5 = 2.0$  (the manner of calculating  $k_1$ ,  $l_1$ , and [HCO<sub>3</sub>] values is given at the end of Theoretical section, I).

For the K region of the fresh kidney  $\epsilon$  is therefore 137 and  $\eta$  is 92.

TABLE VIII  
Values Derived from Table III

$k$	$l$	"c"	$\frac{V}{V}$ Volume of all water in proximal tubules $V = 10$ for fresh tissue	$\eta$	$\epsilon$	$l_1$	$\Sigma d_1$	$k_1$	$k_1$ from Equation 5
6	82	192	1.13	77	111	11.3	13.0	111	124
18	94	216	1.07	56	114	25.5	28.7	135	136
30	106	240	1.09	70	128	25.6	28.8	147	148
60	136	300	1.04	75	131	47.2	51.4	177	178
90	166	360	1.02	76	146	67.0	71.2	214	208
120	196	420	1.01	84	136	95.6	101	236	238
150	226	480	0.99	66	139	130	137	277	268
180	256	540	0.98	79	158	142	149	310	298
210	286	600	0.98	89	144	173	181	328	328

All concentrations are given as mM per litre.

$\Sigma d_1$  is obtained by assuming the small bicarbonate concentration to behave like chloride and adding its value then to  $l_1$ . Calculations of  $l_1$  and  $k_1$  are described in text (at end of Theoretical section, I).

$\eta$  and  $\epsilon$  are calculated from equations of Boyle and Conway (1).

$$\eta/V = c - k_1 - \Sigma d_1$$

$$\epsilon/V = k_1 - \Sigma d_1$$

(c) Potassium Accumulation in Isotonic Mixtures (Causing Large Volume Increases)

The volume of the cell water is governed by the equation (1, 2)

$$V^2(c^2 - 4\sum kd) - 2\eta Vc + (\eta^2 - \epsilon^2) = 0 \quad (6)$$

whence

$$V = \frac{\eta c}{c^2 - 4\sum kd} + \frac{\eta c}{c^2 - 4\sum kd} \times \sqrt{\left\{1 - \left(1 - \frac{\eta^2}{\epsilon^2}\right)\right\} \left(1 - \frac{4\sum kd}{c^2}\right)} \quad (7)$$

For the immersed sartorius muscle  $\eta$  and  $\epsilon$  differ very little so that the equation for  $V$  reduces to

$$V = \frac{2\eta}{c - 4\sum kd/c} \quad (8)$$

and this in turn to:—

$$V = \frac{\eta}{[\text{Na}] + 5.7} \quad (9)$$

since the value of  $c - 4\sum kd/c$  is practically identical with  $2([Na] + 5.7)$  or twice the sum of the concentrations of the non-diffusible constituents in the immersion fluid. Thus for the isotonic mixtures when the external K changes from 10 to 90 mm per litre, "c" being 243 and  $d = 120$ , the value of  $c - 4\sum kd/c$  changes from 223 to 65 and  $2([Na] + 5.7)$  from 225 to 65.

When  $\eta$  and  $\epsilon$  differ very appreciably in the proximal tubules the fuller equation could be expected to give better results. It is a fact, however, that owing to the small increase of  $\epsilon$  with increasing external K concentrations, a simple linear equation again applies very well, though not quite identical in form with the above relation for muscle. It arises in the following way. The value of  $V$  in Equation 7 may be written as:—

$$\frac{\eta}{c - 4\sum kd/c} \text{ multiplying } 1 + \sqrt{\left(1 - \left(1 - \frac{\epsilon^2}{\eta^2}\right)\right)\left(1 - \frac{4\sum kd}{c^2}\right)}$$

Even with the ratio  $\epsilon/\eta$  fixed as the mean value of 136/76 this multiplicand changes only from 2.74 to 2.27 when the external value of  $k$  changes from 10 to 90 mm per litre and  $V$  increases over three times. When, however, a change of  $\epsilon$  from 114 to 155 occurs over the same range of  $k$  then the value of the multiplicand changes only from 247 to 237 or about 4 per cent, and this is further decreased by the slight difference in value between  $c - 4\sum kd/c$  and  $2([Na] + 5.7)$ . The result is that the following relation

$$V = \frac{1.24\eta}{[Na] + 5.7}$$

$$= \frac{94}{[Na] + 5.7} \quad (10)$$

is very well obeyed over a wide range as shown in Table IX.

(The increase of the  $\epsilon$  value from about 114 to 155 as K changes from 10 to 90, and the  $k_1$  value remains approximately at 130 mm per litre, may be deduced from a curve through the  $\epsilon$  values of Table VIII against the  $k_1/k$  ratio, since this latter determines the  $k_1/h$  ratio and so the changes in  $\epsilon$ . These are in turn due to changes in the ionization of the non-diffusible constituents.)

Also, if we consider the relation in terms of  $W_i$ , the actual weight of the whole kidney (immersed), since

$$V = \frac{W_i - 0.42}{0.43}$$

where 0.43 is the water in the proximal tubule cells of the fresh kidney (or 1.00 - 0.18 - 0.34), then from Equation 10

$$W_i = \frac{40}{[Na] + 5.7} + 0.42 \quad (11)$$

which describes the lower curve in Fig. 3 for the immersed kidneys without cyanide.

This gives the fuller theoretical reasons for the linear relation of Fig. 3.

The question may here be briefly considered why it is that with  $\eta = \epsilon$  in immersed muscle, with relatively low external K values, the expected increase in  $\epsilon$  with rising  $k$  has not an appreciable effect on the relation

$$V = \frac{2\eta}{c - 4\sum kd/c}$$

There is firstly the fact that the muscle membrane permeability breaks down at a lower K level than the kidney K cells, at about 70 mm K per litre outside,

TABLE IX  
Values Derived from Table V

$k$	[Na]	$\frac{1}{[Na] + 5.7} \times 10^4$	Volume $V$ of cell water in proximal tubules $V = 1.0$ for fresh tissue	$V$ as calculated from Equation 10
10	107	89	91	84
20	97	97	88	91
30	87	108	102	102
40	77	121	114	114
50	67	138	133	131
60	57	159	149	149
70	47	189	177	178
80	37	234	214	220
90	27	306	284	288
100	17	440	367	414

and from the kidney analogy  $\epsilon$  might be expected to change from 1.14 to 1.45, an increase of 1.28 times; hence from the full equation there would be a  $V$  value only 6 per cent greater than that from Equation 8 or 9. It would appear also that  $\epsilon$  in immersed muscle does in fact alter much less than the above amount, partly because the ionization of the non-diffusible constituents within it changes less with rising pH than the renal K cells and partly because the increase theoretically expected is offset by slight losses of non-diffusible constituents (2).

#### (d) Potassium Accumulation and the Donnan Equilibrium

$$k \times l = k_1 \times l_1 \quad (12)$$

or the product of the potassium and chloride concentrations within the cell water of the K region should be equal to that outside (strictly it is the activity products which should be equal, but for such univalent electrolytes and a  $\mu$  value in the cells probably not much different from that of the external

solution the concentration products may be expected to differ but little on each side of the membrane).

Table X (data from Table VIII) shows the relation of the ion products. It will be seen that from a  $k$  value of 30 mm per litre onwards the relation applies very well. The result is thus similar to that found for muscle except that in the kidney a slightly higher external K value is necessary before the relation applies exactly. For muscle it was pointed out that the cell membrane in the isolated tissue was inefficient with low external K owing to the high concentration ratios across it. A similar explanation may be advanced for the membranes of the renal K cells, and true equilibrium may not be reached with relatively low K concentrations in the immersion fluid. It may also be considered that at such low external K values the proportion of total chloride

TABLE X

$k$	$\frac{k \times l}{(X 10^{-3})}$	$\frac{k_1 \times l_1}{(X 10^{-3})}$	$\frac{k \times l}{k_1 \times l_1}$
6	0.492	1.25	0.39
18	1.69	3.44	0.49
30	3.18	3.76	0.85
60	8.16	8.35	0.98
90	14.94	14.3	1.04
120	23.52	22.6	1.04
150	33.90	36.0	0.94
180	46.1	44.0	1.05
210	60.1	56.8	1.05

contained in the space outside the K-accumulating region is so high that even slight changes in the estimate of this space have a relatively great effect on the estimated Cl content as used for the Donnan product.

### *III. The Identification of the K and Na Regions of the Nephron as the Proximal and Distal Convoluted Tubules*

The existence of a relatively large K region of the kidney impermeable to Na, and a smaller region permeable to Na actively extruding it and losing weight, at once suggests that the two regions are the proximal and distal segments of the nephron. In support of this three reasons may now be advanced which would appear conclusive.

1. It has been shown above that when the kidney is immersed in Ringer solution the cells of the K region (like skeletal muscle fibres) can be greatly swollen in accordance with theoretical expectation, when the Na of the solution is largely replaced by K. No such swelling, but rather a decrease occurs with the cells of the Na region. When the kidney in turn is perfused with such

Ringer solutions containing high K and low Na concentration swelling of the kidney is observed, and here while no increase in volume of the Na cells should occur the relatively large reduction in volume observed in the immersed tissue is not to be expected. This is so because a supply of NaCl is being maintained from the lumen side (though with the high K solution its concentration is reduced below the normal). When after some hours' perfusion the fluid is suddenly replaced by suitable fixative, and the tissues examined histologically (to be described in another paper) the cells of the proximal tubule are found much swollen and those of the distal tubule a little shrunken. The following is typical of the result obtained. Taking the mean cross sectional areas of the proximal tubule minus that of the lumen to be 100 in the control kidney, or kidney perfused with normal Ringer solution (total strength of 243 mm per litre), then for a series of measurements (7 to 10 in each case) there is a mean change from 100 to 135 on perfusing with Ringer solution containing 70 m. eq. K per litre and 47 m.eq. Na per litre. The cross-sectional area of the

TABLE XI

	Dimensions mm.	Dimensions	Relative volume per cent	Relative lengths per cent
Proximal tubule.....	3.80 × 0.075	84.8	51	
Distal tubule.....	2.50 × 0.030	8.9	34	
Glomerulus.....	0.125 × 0.100	3.5	1.7	
Collecting tubule.....	0.70 × 0.028	2.2	9.5	
Neck segment.....	0.10 × 0.030	0.4	1.4	
Intermediate segment.....	0.20 × 0.022	0.3	2.7	

distal tubule changes from 106 to 91, or shows some reduction instead of an increase.

If the increase in length of the proximal tubule is similar to the increase in cell diameter, this would mean a total volume increase of 58 per cent or 70 to 80 per cent increase in the cell water. This accords with theoretical expectation for the swelling of the K region, and it may be concluded that the proximal tubule is the K region investigated above. Such direct evidence is in turn supported by the two following points.

2. The relative size of the K region to the whole renal tissue in the isolated kidney is the same (within the limit of error involved) as Huber's measurements of the proximal tubule (Table XI), and there is a similar correspondence between the Na region and the distal tubule, though, owing to its small size and the large intercellular volume, the margin of error involved for the distal tubule is relatively greater.

Huber's measurements (12) show that the proximal tubule in the isolated immersed tissue is 85 per cent of the whole nephron and the distal tubule

9 per cent. If the glomerulus be excluded from the nephron the figures are 88 and 9 per cent respectively.

For the kidney immersed in Ringer solution with ordinary K content (and total molecular concentration of 243 mm per litre) the weight falls to 0.74, the total solids being 0.16 and the Na ratio 0.26. Taking the Na ratio to measure the total water in the intercellular spaces plus that in the Na cells, the water of the K region is  $0.74 - 0.16 - 0.26$  or 0.32. If we take the inulin ratio of 0.23, in the cold, to measure the intercellular space, the total water in the nephron is 0.35, so that the water of the K region is 91 per cent of the water in the cells of the whole nephron tissue, and the K tissue in turn may be presumed to have this proportion also of the whole nephron. The water of the Na region would then be 9 per cent of the total nephron water.

As considered above the Na region decreases much in volume on immersion, the Na ratio falling from 0.39 to 0.26 with a marked loss in weight of the kidney. If we subtract the inulin ratio from these figures the water of the region would change from 0.16 to 0.03. The question arises then—is there any direct evidence that a diminution of this order occurs in the size of the distal tubule? This we can provide in conjunction with Huber's data. As given above the relative cross-sectional area of the cells of the proximal and distal tubules after perfusing with Ringer solution of ordinary type is 1.0 to 1.06, but from Huber's data after immersions the ratio of the diameter is 1.0 to 0.4 and the area therefore 1.0 to 0.16. This change is of the expected order.

3. The experiments of Walker, Hudson, Findley, and Richards, (13) show that while the Cl content of the flowing urine in the frog's kidney remains unchanged along the proximal tubule (samples being obtained by cannulating the nephron), it falls steeply when the distal tubule is reached, and with it no doubt the Na ion concentration. The distal tubule at least must be freely permeable to Na and Cl, and the curve of concentration with distance from the glomerulus suggests a marked difference in behaviour to the Na ion in the two segments, for the Cl concentration remains unchanged along the proximal tubule and falls steeply from the beginning of the distal tubule. If then, there are two regions of the nephron comprising practically its entire length, the larger impermeable to Na and the other permeable, this latter must be the distal tubule, and clearly the other very large segment can only be the proximal tubule.

#### *Interpretation of the Hemoglobin, Inulin, and Na Ratios*

The average inulin and Na ratios for the active kidney in the cold were found to be 0.23 and 0.26. The question is, what exactly does the inulin ratio measure? The indications are that, at least in the cold, it measures the interspaces of the frog kidney, in which may be included the lumen volume of the nephrons and glomeruli. The glomerular membrane as is well known from

the work of the Richard's school is freely permeable to inulin, and over 24 hours inulin could be expected to reach an equilibrium value in the lumen spaces.

That no appreciable amount of inulin is adsorbed is very probable from the analogy with muscle, where the inulin ratio gives a value as low as 0.09 and it is scarcely possible that appreciable adsorption could occur, when the interspace volume is compared with that from other methods (2). In the cold the effect of cyanide on the kidney is to increase slightly, but scarcely significantly the inulin ratio, this changing from  $0.23 \pm 0.012$  to  $0.27 \pm 0.016$  (giving the standard deviation of the means), whereas the Na ratio changes from  $0.26 \pm 0.014$  to  $0.55 \pm 0.023$ . This indicates that the distal tubules are—in the cold—impermeable, to inulin or so slightly permeable as makes little difference to the results discussed. At room temperature the cyanide effect on the inulin ratio is much greater, changing from  $0.23 \pm 0.020$  to  $0.32 \pm 0.028$ .

Concerning the hemoglobin ratio, this may be taken as measuring approximately the interspaces of the kidney apart from the lumen volume of the nephrons, basing this view on the impermeability of the glomerular membranes to hemoglobin and that diffusion into the lumen from other regions is either debarred or of negligible rate. It may be added that the exact interpretation given of the hemoglobin ratio makes no difference in the general treatment of the results throughout the paper; and the same applies even to the inulin ratio except in so far as this may be used to calculate the water of the Na cells in the active kidney by subtracting from the Na ratio.

#### DISCUSSION

From the experiments described it is clear that the frog's nephron is divisible into two main cellular regions, in one of which K can be accumulated, and without volume increase if the external Na be maintained constant. This region of the kidney contains no appreciable sodium and conversely the other region contains no appreciable potassium (or only of plasma level) while its Na content is at or close to the Na value of the external fluid. These regions have been identified as the proximal and distal tubules (though the K-accumulating region may include the comparatively very small collecting tubule—amounting to 2 to 3 per cent of the nephron). The cell membranes of the first are permeable to potassium but not to the sodium ion. The membranes of the latter are permeable to Na ions and to the smaller K ion (to the latter at least from the blood side). The process of accumulation follows well the theoretical treatment already outlined for muscle, and specially notable is the correspondence of the volume changes with the theoretical requirement. The production of these large volume changes and their measurement is very simple and affords a ready means of testing the underlying theory. In measuring the volume changes and weighing the tissues after immersion no undue pressure

should be exerted, especially on the highly distended tissues, a fixed routine of light surface drying on filter paper being advisable.

*The Effect of Cyanide.*—For the isotonic Ringer solutions where K is substituted for Na with consequent large volume changes, the effect of cyanide is strikingly shown by means of the linear relation of volume to the reciprocal of the external non-diffusible constituents as appears in Fig. 3. Cyanide does not change the slope of the line up to an external concentration of 70 mm per litre. This means that it affects neither the differential permeability of the K cells nor does it affect their volume. It appears, however, to render their membrane system less resistant to high distension, for with cyanide the differential permeability breaks down when the cell water has about doubled, whereas without cyanide this does not happen until an increase of nearly four times occurs. While not affecting the volume nor differential permeability of the K cells (up to high limits of distension) cyanide has a great effect on the renal volume as a whole as shown in Fig. 3 arising from the fact that it converts the active contraction into a slow distension. The line through the points after 24 hours cuts the ordinate at 0.62. This will include 0.18 (mean dry weight of the cyanide kidney) leaving 0.44, and subtracting the inulin ratio of 0.23 as representing the total intercellular spaces, this leaves 0.21, so that the water of the Na cells instead of decreasing markedly from the fresh value of 0.16, as in the active kidney, has somewhat increased. After 48 hours this water in the cyanide kidney increases to 0.27. Now such distension agrees with the conclusions drawn that the cells are quite permeable to Na and K ions, but that in their active state they are capable of keeping Na and water steadily extruded and decrease in volume. When normally functioning in the animal their volume would appear to be associated with a steady influx of Na ions from the lumen side.

It will be seen that the fortunate appearance of a practically exact linear relation of renal volume to the reciprocal of ( $[Na] + 5.7$ ) throws much light on the nature of the cyanide effect, and also confirms the measurement of the renal tissue outside the K region.

For such passive accumulation of K in the proximal tubules the question arises as to its possible relation with the normal active excretion of potassium or other substances—a question arising not only for the frog's kidney, but as a general one for active renal excretion. That it has an important relation may be considered likely, but a treatment of such implications will be deferred until later, the further discussion here being confined to a consideration of the absorption of water from the urine as it flows down the proximal tubules in the normal animal.

#### *The Question of Water Absorption in the Proximal Tubule of the Frog's Kidney*

The proximal segment in the immersed frog's kidney comprises 85 per cent of the volume of the whole nephron, and it would appear to be about 70 per

cent of the nephron volume when normally functioning in the animal. Whether in the latter case water is absorbed or not is of special significance if only from the analogy with the mammalian kidney, in the proximal tubule of which about 80 per cent of the whole water absorption in the nephron would occur if we explained the glucose increase after phlorhizin as due to the absorption of fluid.

It has been shown that no diminution of chloride occurs down the proximal segment in the frog's kidney (13) nor does any significant change of pH occur in the same region (14).

If the cells of the proximal tubule are impermeable to Na ions, as shown for the immersed kidneys, there can be no appreciable absorption of water, for if water be absorbed, chloride and bicarbonate must follow in similar concentration to maintain the lumen concentration constant, and sodium is the only cation available with the required concentration to accompany the chloride and bicarbonate ions.

Such an argument might be met by the objection that some section only of the proximal tubule cells need be concerned with the Na absorption, but since the process (or apparent water absorption) is progressive down the tubule, we should need to picture Na cells scattered between the K cells, but from volume considerations they could constitute at most only a small fraction of the whole. When the proximal tubule is largely swollen by low Na and high K perfusions or immersions they might be expected to appear as localised constrictions, but so far we have noticed none such, and the picture of such scattered Na cells seems a highly unlikely one. Without unduly labouring the question it will be seen that the facts of K and Na exchanges are against the view of water absorption in the proximal tubule.

On the other hand, we must conclude that if no water be absorbed in the proximal tubule, then the increase of glucose concentration after phlorhizin is necessarily an active excretion. Such has been regarded as inconceivable (*e.g.* Walker and Hudson (20); Walker *et al.* (18)) whether for teleological reasons, or otherwise. But if we consider the reverse process of glucose secretion into the blood from the urine, and this to depend on phosphorylating and dephosphorylating mechanisms (in accordance with Lundsgaard's views (15, 16) or modified versions) and an orientation of cell metabolism such as developed by Danielli (17), and long entertained in similar form in this laboratory, it would seem by no means inconceivable that an inhibitor of carbohydrate metabolism unequally concentrated within the cell, might reverse the direction of glucose passage.

Besides the phlorhizin effect, the collection rates of fluid down the tubule are held to support the view of water or fluid absorption. This is considered below.

*The Proximal Tubule of the Mammalian Kidney.*—Our investigations concerning the K equilibrium etc. have not been extended to the mammalian

kidney, but provisionally we may assume that the proximal tubule has the same permeabilities as found for the amphibian kidney. If this is so, and no water be absorbed down the proximal tubule, it is an essential requirement that the Na ion concentration should not change, but correspond to that in a plasma ultrafiltrate. This is the result found by Walker *et al.* (18).

Concerning the collection rates found down the tubule the following very simple relations may be firstly considered. If, on cannulating any part of the tubule, and registering the flow, we write " $G$ " for the rate of glomerular filtration throughout the collection, " $S$ " the amount of fluid absorbed as calculated from the glucose concentration after phlorhizin (assuming this to be due to water absorption), and " $D$ " an extra amount of fluid absorbed not included in " $S$ " (owing to possible back diffusion of glucose) then the difference in rates of collection from two tappings at different sites appears as:—

$$(G_1 - S_1 - D_1) - (G_2 - S_2 - D_2)$$

which may be written:—

$$(G_1 - G_2) + (S_2 - S_1) + (D_2 - D_1)$$

(the symbols  $G_1$ ,  $S_1$ , and  $D_1$  referring to the tapping nearer to the glomerulus).

If we consider these symbols as representing average figures in a number of collections, then fluctuations in  $G$ , and in resistance due to the collecting pipettes, etc. will tend to disappear.  $G_1$  will then be necessarily, if only very slightly, greater than  $G_2$  and  $D_2$  will tend to be greater than  $D_1$ , so that  $(S_2 - S_1)$  will give a minimal mean value for the water absorption between the sites of collection. The actual mean water absorption should be greater. This argument is independent of the question of the resistance to flow offered by the pipettes, or as to whether the collection rates correspond to normal rates of flow down the tubules. With a sufficient number of results it should answer decisively the question as to whether the glucose increase after phlorhizin is due to fluid absorption. This should amount to more than about 60 per cent down to half the length of the proximal tubule. As reproduced in the first series of Table XI Walker *et al.* (18) have summarised their own data for rats obtained in experiments of great manipulative elegance. It will be seen that they find 1.7 c.mm. for the first fifth, 1.1 for the second, and 0.8 c.mm. per hour for the third fifth. This would appear to support their views, but on examination the apparent effect is entirely due to one very aberrant figure (No. 12 of their series), the deviation of which from the mean is about four times the standard deviation for its group. This, included in the arithmetical mean of the first three figures gives the high value for the collection rate from the first fifth of the proximal tubule. The medians from the same data are shown in the second series of Table XII, and no effect of diminished rates down the tubule is apparent. The next two series give the means and medians for the guinea pig,

for which glomerular collection data are also available, and it will be seen that no diminution appears on passing from the glomerulus to the middle of the proximal tubule or about as low as it has been found feasible to collect the urine. Applying a stricter statistical treatment the correlation between the collection rates and distance down the tubule (taking the glomerular collection as at zero distance) is  $-0.03 \pm 0.21$  for the twenty-three guinea pig results, and no decrease with distance can be deduced from the results. For the twenty-three rat experiments (omitting No. 12) the correlation is  $-0.11 \pm 0.21$ , and the regression equation shows a very doubtful fall of 17 per cent in the collection rates midway down the proximal tubule, which may be compared with the expected fall of more than 60 per cent. The correlation of the whole forty-seven collection rates from rats and guinea pigs with tubular

TABLE XII  
*Average Collection Rates of Tubular and Glomerular Urine*

Animal	Nature of average	Glomerulus	Proximal tubule		
			1st fifth	2nd fifth	3rd fifth
Rat	Mean	—	1.7 (3)	1.1 (8)	0.8 (13)
	Median	—	0.9 (3)	1.0 (8)	0.8 (13)
Guinea pig	Mean	0.7 (6)	0.8 (3)	0.8 (7)	0.7 (6)
	Median	0.6 (6)	1.0 (3)	0.7 (7)	0.6 (6)

Data taken from Walker *et al.* (18).

distance is  $-0.18 \pm 0.15$ , and for the forty-six results omitting the aberrant value it is  $0.05 \pm 0.15$ . On the other hand there are high correlations (order of  $+0.80$ ) between creatinine (or glucose after phlorhizin) concentration ratios and the tubular distance. In such results commented on above, the total variability of the collection rate is not high, being only 40 to 45 per cent as a coefficient of variation, and it will include the variability of glomerular flow, pitette resistance, etc. It is easy to show that changes in these variables from tubule to tubule, with an underlying relation of distance down the tubule to amount of water absorption cannot then account for the low correlations observed between collection rates and sites of collection. They would at most reduce it from  $-1.00$  to  $-0.75$  or from  $-0.80$  to about  $-0.60$ . The position remains essentially the same if a logarithmic relation of collection rate to distance is considered instead of a linear.

If similar figures be examined for the frog (20) there is not the same exact measurement of tubular distance as for the mammal, but for the fifteen results listed for the proximal tubule collections, the mean and median for the seven rates down to the first half of the tubule are 0.37 and 0.35 c.mm. per hour,

and for the seven collections at the end 0.46 and 0.39 c.mm. per hour, showing a slight but scarcely significant *increase*. For the frog it is true that the rate of glomerular collection—as judged in this series by three experiments—is 0.72 c.mm. per hour (median value). Such a difference may be possibly due to the higher pressures at the head of the tubule (the glomerulus being here succeeded by a short, but comparatively narrow neck). The difference is no doubt also emphasised by the selection of the most suitable glomeruli for puncture; that is, the most distended.

It may be reasonably concluded that the observed collection rates do not support the view that fluid is absorbed in the proximal tubule, but rather the contrary that there is no fluid absorption therein; and this agrees with the deductions from the permeability studies described in the present paper.

#### SUMMARY

1. In a manner similar to that of the sartorius muscle, the isolated kidney of the frog can accumulate K against a gradient to upwards of three times its normal concentration.

2. The K-accumulating region is identified as the proximal tubule, which in the isolated tissue immersed over 24 hours in the cold (2–3°C.) amounts to about 90 per cent of the nephron minus the glomerulus. In the fresh tissue it constitutes about 70 per cent.

The cells of the proximal tubule are impermeable to Na, but freely permeable to K and Cl.

3. The distal tubule in the isolated kidney does not accumulate K over the external concentration. The cells are permeable to Na which they actively extrude. This extrusion of Na goes parallel with a loss of osmotically associated water amounting to about 15 per cent of the weight of the fresh kidney, but varying somewhat with the conditions.

4. The accumulation of K in the proximal tubules is in accordance with the equations established for the sartorius muscle, and, as theoretically expected, there is no volume increase (but rather a small decrease) with the large accumulations, when the external Na concentration is maintained throughout.

5. With K accumulation in isotonic mixtures large volume changes occur as K is progressively substituted for Na. Over the range of external K concentration of 10 to 100 mm per litre the weight of the whole kidney changes to 2.5 times and the water of the cells of the proximal tubules increases to over four times. Up to an external K value of 90 mm per litre the mean weight of the kidney shows a linear relation when plotted against the reciprocal of the Na concentration plus the small glucose and Ca concentration. This relation is interpreted theoretically.

6. The effect of cyanide in the isotonic mixtures is to prevent the contraction of the distal tubules and to cause swelling of the same. It does not affect the

volume, volume changes, or differential permeability of the proximal tubule. At the same time the membranes of the proximal tubule cells lose their characteristic permeability at a lower level of distension in the presence of cyanide.

7. The mean Na ratio for the kidney after 24 hours' immersion in the cold is  $0.26 \pm 0.014$  (giving standard deviation of mean). The ratio is defined as  $\frac{\text{Na/kg. of original tissue}}{\text{Na/kg. external fluid}}$ .

For the fresh kidney the mean ratio is  $0.39 \pm 0.006$ .

8. The mean inulin ratio (28 observed in the cold) is  $0.23 \pm 0.012$  and the same value for 10 observed at room temperature. At room temperature—2 hour immersion—the ratio is increased by cyanide to a mean of  $0.32 \pm 0.028$ , but only a slight increase is caused by cyanide in the cold.

9. The mean hemoglobin ratio after 24 hours' immersion in the cold is  $0.17 \pm 0.004$  and is unaffected by cyanide.

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# ANOXIA AND BRIGHTNESS DISCRIMINATION\*

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## I

### *Nature of Work*

It is the commonest of observations both in actual flying and in pressure chamber experiments that lights become dim at high altitudes and then brighten on return to low altitudes. These events are due to the absence and presence of an adequate oxygen supply, as can be shown by their easy elimination when sufficient oxygen is available.

The phenomenon itself has been demonstrated quantitatively as a rise in threshold of the dark-adapted eye during anoxia by McFarland and Evans (1939), by McFarland and Forbes (1940), and by Wald, Harper, Goodman, and Krieger (1942). (*Cf.* also Bunge, 1936, and Fischer and Jongbloed, 1936.) In addition McFarland and Halperin (1940) found that visual acuity is affected by decreased oxygen tensions.

Brightness discrimination and visual acuity are closely allied visual functions and it is not surprising that Schubert (1932-33) and Gellhorn (1936) detected an influence on brightness discrimination, though they recorded sharply conflicting results. However, McFarland, Halperin, and Niven (1944) have definitely shown that brightness discrimination is impaired by anoxia, and have explored the effect over the significant range of cone vision.

Our own work—completed in 1942 but only now available because of its origins—confirms the findings of McFarland, Halperin, and Niven. Our experiments were made somewhat differently from theirs; theirs cover a greater brightness range, but in most of their work a single low oxygen concentration was compared with room air. We compared seven different oxygen concentrations with room air, at three moderate brightnesses involving cone vision only.

## II

### *Apparatus and Procedure*

If  $I$  is the light intensity of a uniform field of vision, and  $\Delta I$  is the light intensity which needs to be added to a part of this field so that the particular

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shape of the addition may be correctly recognized by an observer, then the fraction  $\Delta I/I$  is a measure of the brightness discrimination of the observer for the prevailing conditions. The larger the fraction  $\Delta I/I$  the greater is the contrast necessary, and the poorer is the brightness discrimination.

In the present experiments the visual field is a large circular area in front of which the observer sits comfortably and becomes adapted to its particular light intensity. By means of a shutter he can project the illuminated image of a broken circle (Landolt Ring or C) on the field for 1/5 of a second. The position of the break in the circle is set by the operator, and it is the task of the subject to discover this position by gradually increasing the brightness of the broken ring.

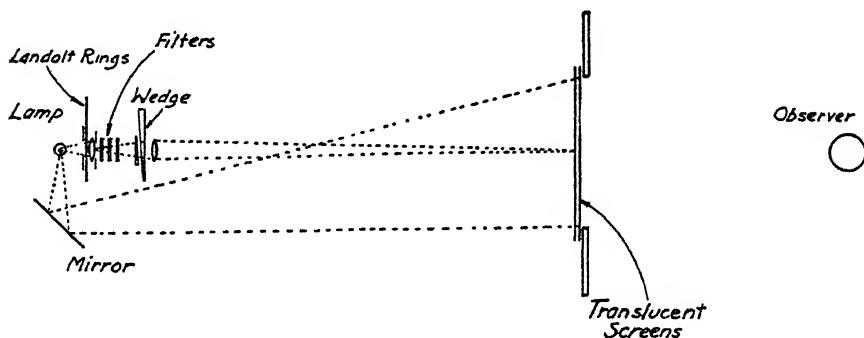


FIG. 1. Diagrammatic top view of experimental arrangements. The adapting light  $I$  covers the whole screen; while the added light  $\Delta I$  is in the form of a projected broken circle. Both come from the same lamp, but are separately variable.

The precise arrangements in the dark room may be seen in Fig. 1 which is a diagrammatic top view. The lamp, in a light-tight container, is a 100 watt concentrated filament projection bulb; it is operated through a voltage regulator, and furnishes both the background field illumination  $I$  and the added illumination  $\Delta I$  for the projected C. The background beam, after passing through neutral and color filters, is reflected by a mirror on to the translucent screen composed of a sheet of flashed opal glass and a sheet of ground glass. The background field is 60 cm. in diameter, and since the subject sits 125 cm. in front of it, it subtends a visual angle of  $27^\circ$ .

The added illumination in the form of the Landolt broken ring is projected by two lenses from an opaque slide in which 8 clear rings have been accurately cut, with the break in 8 different positions. The intensity and color of the projected beam is controlled in steps by neutral and color filters, and a neutral wedge and balancer determine the intensity continuously and gradually. The projected Landolt ring subtends  $7.5^\circ$  visual angle, the break being  $1.5^\circ$ . The reason for such a large test object is to place it well over the visual acuity threshold at all illuminations used, and thus confine the study to brightness discrimination alone.

After preliminary experiments we chose three field intensities, approximately 1/1000, 1/100, and 1/10 millilambert, (actually 0.00085, 0.0102, and 0.12 millilambert), thus covering that region of daylight vision in which contrast discrimination varies most rapidly with illumination (Hecht, Peskin, and Patt, 1938). To insure the participation of daylight vision alone even at the lowest intensity, we used red light, secured by inserting Wratten filter No. 29 in both light beams. The light intensities, filters, wedges, etc. were carefully calibrated with the Macbeth illuminometer and a Martens polarization photometer.

Conditions corresponding to high altitudes are achieved by having the subject breathe from tanks containing mixtures of oxygen and nitrogen, whose concentrations vary between 9 and 16 per cent oxygen. The subject breathes through an oxygen-therapy mask, having a 2 liter rubber bag as reservoir. Rebreathing is prevented by a valve between mask and bag, and expiration is through a flutter valve to room air. The rate of flow is controlled by a gauge and varies from 8 to 16 liters a minute, depending on the request of the subject.

After adequate adaptation to the light, five successive determinations of  $\Delta I/I$  are usually made with the subject breathing room air without a mask or compressed air from a tank through a mask. A specific oxygen mixture whose concentration is not known by the subject is then supplied to the mask, and an equilibrium period of 15 minutes allowed. Determinations of  $\Delta I/I$  are then begun, and continued until five successive measurements show that equilibrium has been reached. This frequently means no more than five or six trials, but occasionally it involves as many as twenty. In succession, various oxygen mixtures are tested in this way, and sometimes the session is concluded with a repetition of room air. We found the latter to agree with the initial reading, and therefore did not make it regularly.

### III

#### *Brightness Discrimination*

Seven men and one woman served as subjects. With the exception of S. H. (51 years) their ages were between 17 and 25 years.

The data are given in Table I where for each subject, at each background brightness and each oxygen concentration there is listed the increase in  $\log \Delta I/I$  required as compared to the room air performance. For easy understanding, the significant aspects of the measurements are shown graphically in Fig. 2. It presents the relation between oxygen concentration and the percentage increase in the contrast fraction  $\Delta I/I$  as compared to room air. It is apparent that at all three background illuminations, decreasing the oxygen concentration causes a rapidly accelerated increase in the amount of contrast difference which can just be recognized. Thus at lower oxygen tensions increasingly larger brightness differences between surfaces become necessary before they can be seen as different.

Three aspects of this visual impairment are to be noted. The first relates to altitude. The altitudes corresponding to the oxygen mixtures used in the

experiments have been calculated<sup>1</sup> and are shown in Fig. 2. From this it is apparent that for these brightnesses the deterioration of contrast discrimination begins at fairly low altitudes, and is definite at 8,000 feet. At 15,000 feet elevation, deterioration is marked, especially at the lowest brightness where a contrast must be twice as great as at sea level before it can be recognized. Evidently for achieving maximum visual capacities at these illuminations, it is

TABLE I  
*Contrast Discrimination and Anoxia*

Background intensity log $I$ <i>milli-lamberts</i>	Oxygen breathed <i>per cent</i>	Increment in log $\Delta I/I$ over room air for								
		S.H.	S.F.	C.D.H.	B.A.	J.A.	D.P.	A.K.	D.D.H.	
-3.07	16.6	0.00	0.01	0.04	0.00	0.08	0.01	0.05	0.12	0.038
	14.8	0.16	0.07	-0.01	0.03	.0.13	0.17	0.11	0.15	0.106
	13.2	0.12	0.02	0.09	0.14	0.17	0.24	0.20	0.10	0.126
	12.1	0.36	0.15	0.19	0.25	0.22	0.33	0.50	0.21	0.267
	11.2	0.32	0.15	0.24	0.20	0.40	0.33	0.51	0.25	0.302
	10.3	0.35	0.14	0.17	0.19	0.24	0.24	0.98	0.36	0.333
-1.99	16.6	0.00	0.07	-0.02	0.05	-0.03	0.02	0.00	0.02	0.013
	14.9	0.06	0.07	0.03	0.04	0.16	0.03	0.01	0.03	0.042
	13.2	0.15	0.07	0.06	0.11	0.17	0.27	0.00	0.12	0.119
	12.2	0.13	0.09	0.14	0.16	0.20	0.27	0.33	0.15	0.184
	11.1	0.21	0.08	0.11	0.29	0.21	0.32	0.48	0.07	0.220
	10.3	0.27	0.19	0.14	0.30	0.27	0.32	0.34	0.24	0.260
-0.92	16.5	0.11	0.02	0.01	0.00	0.00	0.00	0.03	-0.01	0.020
	14.9	0.05	0.04	0.05	0.07	0.01	0.07	0.08	0.04	0.051
	13.2	0.12	0.09	0.07	0.06	0.08	0.09	0.03	0.10	0.080
	12.2	0.17	0.08	0.10	0.16	0.09	0.23	0.09	0.04	0.120
	11.1	0.28	0.15	0.13	0.20	0.12	0.13	0.09	0.04	0.142
	10.3	0.37	0.17	0.17	0.24	0.22	0.23	0.40	0.20	0.250

necessary to supplement with oxygen the air breathed by those flying at even moderate altitudes.

Second, a given increase in altitude causes a greater impairment in contrast recognition at high altitudes than at lower ones. Thus, in going from 12,000 to 15,000 feet the increase in brightness difference required for contrast recognition is twice as great as that in going from 6,000 to 9,000 feet. Above 15,000 feet there is indication of a somewhat less rapid deterioration of brightness discrimination. This may be due to compensation by the body for the anoxia.

<sup>1</sup> In the computations, proper consideration was given to the saturation of the inspired gas mixture with water vapor, a factor which has occasionally been neglected.

Third, though the deterioration in brightness discrimination occurs at all three light intensities, the effect decreases as the illumination increases. The brightnesses included in the data cover most of the intensity range within which

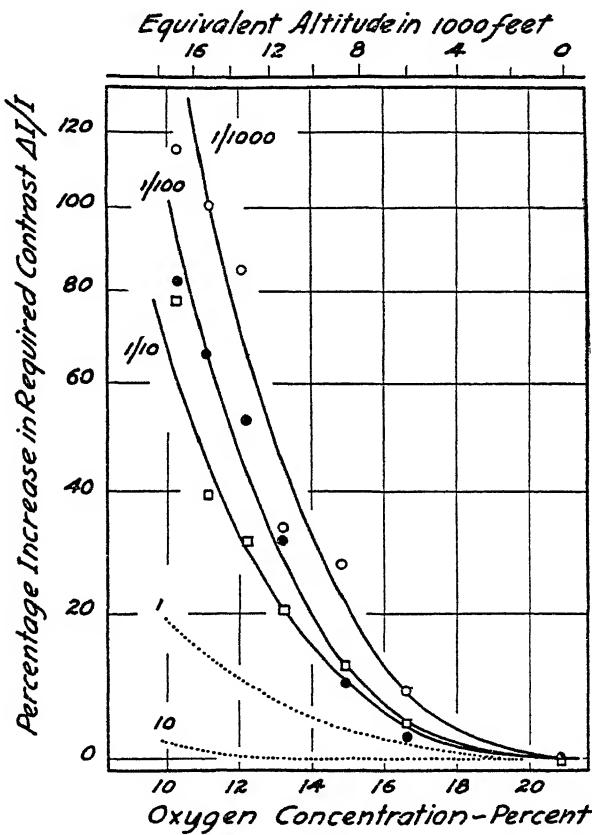


FIG. 2. Oxygen concentration and loss of contrast discrimination at three field brightnesses 0.1, 0.01, and 0.001 millilambert. The percentage increase required in the contrast fraction  $\Delta I/I$  is plotted as ordinates on a logarithmic grid.

contrast discrimination varies. They represent the appearance of objects after sunset, and between dawn and sunrise. For a brightness ten times greater than our highest the deterioration is small but definite, whereas at still higher levels it is negligible. The two dotted curves in Fig. 2 are extrapolations from the measurements (*cf.* section V) and show what would be expected at higher intensities of 1 and 10 millilamberts; these expectations have been realized by McFarland, Halperin, and Niven.

## IV

*Threshold after Dark Adaptation*

Several groups of measurements have been reported on the effect of anoxia on the final dark-adapted threshold of the eye (McFarland and Evans, 1939; McDonald and Adler, 1939; and Wald, Harper, Goodman, and Krieger, 1942). We made similar tests first, to see whether our oxygen mixtures would give comparable results; and second, to compare the effect of anoxia on the dark-adapted cone threshold with that on the rod threshold.

TABLE II

*Anoxia and Thresholds after Complete Dark Adaptation*

The absolute values at room air are given in parentheses in the first line. The other values are the increments in  $\log I$  compared to room air.

Oxygen breathed <i>per cent</i>	Increase over room air in threshold intensity, $\log I$ , micromicrolamberts					
	With violet filter (rod)			With red filter (cone)		
	S.F.	C.D.H.	Average	S.F.	C.D.H.	Average
21.0	(3.01)	(2.43)	(2.72)	(5.87)	(5.39)	(5.63)
16.4	0.00	0.10	0.05	0.07	0.08	0.08
14.9	0.12	0.09	0.11	0.17	0.08	0.13
13.2	0.18	0.40	0.29	0.12	0.12	0.12
12.2	0.35	0.34	0.35	0.27	0.27	0.27
11.1	0.20	0.29	0.25	0.37	0.25	0.31
10.6	0.30	0.40	0.35	0.34	0.25	0.29
9.4	—	0.50	0.50	—	0.42	0.42

For two subjects the thresholds of the completely dark-adapted eye were measured with the Hecht-Shlaer adaptometer (Hecht and Shlaer, 1938). The Corning 511 violet filter was used for the rod threshold; and either the Wratten 88 or 29 red filter for the cone threshold.

The individual measurements are given in Table II. The data refer to a circular  $3^\circ$  retinal field situated  $7^\circ$  nasally. The averages are shown in Fig. 3, which includes as well the results of previous investigators.

From this comparison it follows that our measurements correspond to those of previous workers about as well as they agree with one another. Moreover, it appears that within the limits of experimental error, cone and rod thresholds are affected equally by low oxygen concentrations.

The effects on the absolute cone threshold are slightly larger than for the contrast threshold at the lowest brightness shown in Fig. 2. This is in keeping

with the general trend of the measurements that visual deterioration is more evident at lower than at higher illuminations.

## V

*Contrast Discrimination, Final Thresholds, and Anoxia*

It is possible to show that the quantitative effects of anoxia on contrast discrimination are intimately related to the effects on the final dark-adapted

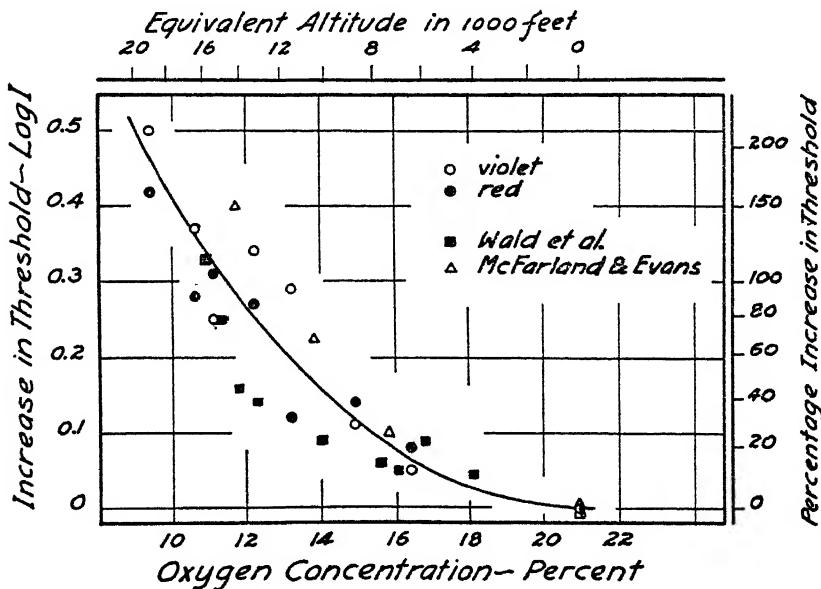


FIG. 3. Visual threshold for cone and for rod vision as influenced by the concentration of inspired oxygen. The threshold is compared to that at room air, and it is apparent that the effect on cone (red light) and rod (violet) vision is about the same.

thresholds reported in the preceding section. To bring this out we need to recognize the well established relation between light intensity and contrast discrimination.

For convenience in showing the data in Table I and Fig. 2, we presented the changes in the contrast fraction  $\Delta I/I$  during anoxia relative to the normal values in room air. The actual numerical magnitudes of  $\Delta I/I$  in room air for eight subjects are given in Table III, so that from them the numerical values may be derived for all other altitudes in Table I for each subject.

Our interest here lies only in the average results, and in Fig. 4 these are plotted against the background intensities. Through the data has been drawn the theoretical curve for the dependence of the intensity discrimination,  $\Delta I/I$ ,

on the light intensity,  $I$  (Hecht, 1935). It is to the point that McFarland, Halperin, and Niven (1944) found their measurements, which cover a greater

TABLE III  
*Contrast Discrimination in Room Air*

Background intensity log $I$	log $\Delta I/I$ for								Average
	S.H.	S.F.	C.D.H.	B.A.	J.A.	D.P.	A.K.	D.D.H.	
<i>millilamberts</i>									
-3.07	-0.29	-0.53	-0.71	-0.43	-0.44	-0.38	-0.43	-0.61	-0.477
-1.99	-1.07	-1.31	-1.31	-1.18	-1.10	-0.96	-1.22	-1.25	-1.175
-0.92	-1.70	-1.79	-1.82	-1.74	-1.59	-1.56	-1.73	-1.74	-1.709

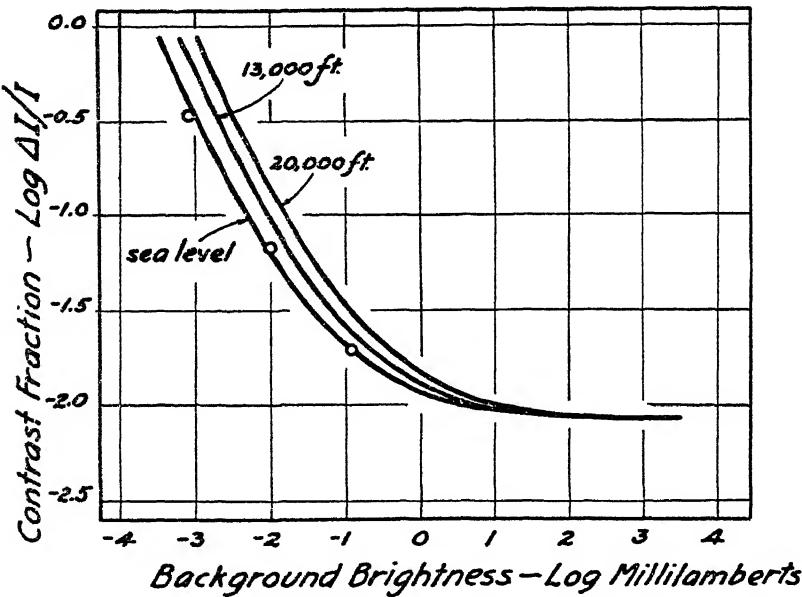


FIG. 4. The relation between just perceptible contrast  $\Delta I/I$  and the prevailing  $I$ . The curves are theoretical and are from equation (9). The effect of altitude (or oxygen lack) is merely to translate the curve along the abscissas.

range of illuminations than ours, to be described with fine precision by this same theoretical equation.

We have shown in the preceding section that the cone threshold is increased during anoxia. What this means is that the top of the curve in Fig. 4 is shifted to the right to higher and higher intensity levels. It is reasonable to suppose that not merely the top of the curve, but the curve as a whole is

shifted and that this is the basic visual effect of anoxia. Such a shift of the whole visual function along the intensity axis has already been found by McFarland and Halperin for visual acuity, and by McFarland, Halperin, and Niven for brightness discrimination. In fact, they were able to show that for a given oxygen concentration the intensity shift for brightness discrimination is exactly the same size as the shift in cone threshold during dark adaptation.

In Fig. 4 we have drawn these shifted curves for three altitudes, using the average threshold measurements of Fig. 3. It is apparent that though the horizontal intensity distance is the same between any two curves, the vertical distance becomes less as the intensity increases. In other words, though anoxia actually affects the whole intensity discrimination function at all levels, the effect is considerably more apparent at lower intensities than at high.

One can find the precise horizontal displacement of the curves from the

TABLE IV  
*Horizontal Displacement in Log I of the  $\Delta I/I$  Function at Different Oxygen Concentrations*

Oxygen concentration per cent	Shift in log I
21.0	0.000
16.6	0.037
14.8	0.107
13.2	0.181
12.1	0.309
11.1	0.360
10.3	0.474

measurements of contrast discrimination themselves. The data in Table I and in Fig. 2 give the vertical displacement of the  $\Delta I/I$  curves at different oxygen concentrations for the three light intensities. These vertical displacements can be converted into horizontal displacements by dividing the vertical increment at any intensity by the slope of the curve at that particular intensity. On a large scale plot of Fig. 4 we measured the slopes of the curve at the three experimental intensities. They are 0.752, 0.588, and 0.465 going from the low to the high intensity.

For each oxygen concentration we have in Table I a vertical displacement at each intensity. Dividing each displacement by the appropriate slope, and averaging the three we get the horizontal displacement for each oxygen concentration. These averages are given in Table IV and thus form the basic description of the effect of oxygen lack on brightness discrimination. From them one can determine the horizontal shift of the  $\Delta I/I$  curve for any altitude, after which one can then easily determine the vertical shift in order to get the change in  $\Delta I/I$  at any value of the intensity. Note that in Table IV the hori-

zontal shift for 11.1 per cent oxygen is 0.360 log unit. This agrees well enough with the value of 0.356 log unit for 10.8 per cent oxygen found by McFarland, Halperin, and Niven to serve as independent corroboration of the measurements.

## VI

*Oxygen Lack and Retinal Chemistry*

The dislocation of the visual system during oxygen lack is most likely an indication of the similar impairment of many other parts of the nervous system. Indeed it has been the common supposition (Gellhorn, 1942) that the changes in visual function are merely an index of the impairment of the central nervous system. While this may be true, it has not been established; and there are some indications that the retina itself may be the seat of the anoxic changes shown in vision.

One such sign is the high oxygen requirement of the retina. The rat retina consumes 31 c. mm. O<sub>2</sub> per mg. per hour. This is the highest rate of oxygen consumption of any tissue recorded by Warburg (1927), the nearest competitor being kidney with an oxygen consumption of 21 c. mm. per mg. per hour.

A related piece of evidence is that when flyers are subjected to centrifugal forces several times gravity they become temporarily blind before they become unconscious. It is thus likely that the retina is more sensitive to oxygen lack than the cortex. Actually Craik and Vernon (1941) have shown that the visual threshold can be changed markedly merely by cutting off the oxygen supply to the retina.

Whether the effects of anoxia on the retina depend on the impairment of its specifically nervous elements like bipolar and ganglion cells, or of the rods and cones themselves cannot now be decided. Any consideration of the effect of anoxia, however, needs to begin with the theoretical formulation of visual chemistry which has served as an accurate description of brightness discrimination, visual acuity, and other visual phenomena (Hecht, 1937).

We start with two established facts. One is that the rods contain a light-sensitive substance, visual purple, and the cones contain a similar substance, visual violet, both of which absorb light and are changed chemically by it. The other fact is that these photosensitive substances are constantly replenished, probably by the combination of some of their photoproducts with additional materials like vitamin A supplied by the retina and ultimately by the circulation.

Let the concentration of photosensitive material in the cones in the dark be  $a$ . Let  $a-x$  be its concentration at the intensity  $I$  after the eye has become adapted to the light; therefore  $x$  is the concentration of photoproducts formed

which remain in the cones to be used for the regeneration of the sensitive material. The rate of photochemical change is

$$\frac{dx}{dt} = k_1 I(a - x)^m \quad (1)$$

where  $k_1$  is the combined absorption and velocity constant and  $m$  represents the order of the reaction, and may be 1, 2, etc. The rate of regeneration from the photoproducts is

$$-dx/dt = k_2 x^n \quad (2)$$

where  $k_2$  is a velocity constant and  $n$  is the order of the reaction. At light adaptation to the intensity  $I$  the two rates become equal, and a photostationary state is reached. Setting equation (1) equal to equation (2) and solving for  $I$ , we get

$$k_1 I / k_2 = x^n / (a - x)^m \quad (3)$$

which represents the relations between light intensity and the concentrations of photosensitive substance and photoproducts at the stationary state.

The photostationary state during light adaptation appears superficially like an equilibrium, in that its components remain constant. However, it is not a true equilibrium because it can be maintained only by the continuous addition of light. Without the light, the back-reaction would at once increase the concentration of sensitive material. And without the back-reaction the light would destroy all the photosensitive material and vision would disappear. The balance between the action of light and the regeneration reaction insures a steady concentration of sensitive material which thus absorbs light at a regular pace and converts it into chemical action at a rate set by  $k_1 I(a - x)^m$ , so that we continue to see the light as long as it shines.

Equation (3) of the photostationary state in the retina has served as a quantitative description for a variety of visual functions as they vary with the adapting intensity of the light. Intensity discrimination, visual acuity, flicker perception, instantaneous threshold, all vary in a similar way with brightness and all fall into a comparable pattern when expressed as functions of concentration at the stationary state (Hecht, 1937).

In setting up equations (1) and (2) we introduced the fewest components required for quantitative description and explanation. The result is that equation (3) involves light intensity and concentration with only the most necessary constants to connect them with known chemical and physical laws. However, in making visual experiments one does not measure concentrations of sensitive materials; instead, one has to measure some physiological property like brightness discrimination or visual acuity which is the expression of nerve impulses resulting from the chemical transformation of the sensitive pigments

by light. Therefore there is needed an explicit formulation for the conversion of photochemically effective light into magnitude of visual function. In the past this relation has usually been assumed as one-to-one and has been implicitly included in the constant  $k_1$ . This procedure has been adequate because the physiological conditions of the organism as a whole have been maintained constant during such measurements, and therefore the relation between chemical change and physiological magnitude has been assumed constant.

The situation during oxygen deficiency, however, demands a more explicit recognition of this relation because there is every likelihood that the physiological conditions inside the organism are changed, and the magnitude of the change may well affect the transformation of chemical change into physiological function. Let us still assume a simple relation between the two but let us merely designate a factor for it. Let the conversion of chemically effective light into visual function be governed by the factor  $\alpha$ . This factor must then be included in equations (1) and (3) whenever these represent visual properties rather than merely concentrations. The simplest meaning to attach to  $\alpha$  is that of a linear conversion factor from light absorbed to number of impulses leaving the particular sense organ, or ultimately reaching the cortex. One may then write

$$di/dt = \alpha dx/dt = \alpha k_1 I(a-x)^m \quad (1a)$$

as representing the rate of impulse production  $i$  during the stationary state, or more generally the magnitude of any physiological counterpart of the photochemical change. In the same visual terms the stationary state equation becomes

$$\alpha k_1 I/k_2 = x^n/(a-x)^m. \quad (3a)$$

Under normal physiological conditions the conversion factor  $\alpha$  is constant and merges with  $k_1$  to form one constant. But under changed physiological conditions such as oxygen deficiency  $\alpha$  is bound to change, even though both  $k_1$  and  $k_2$  probably remain constant. With lack of oxygen the conversion factor is surely smaller than normal, and  $\alpha$  decreases. Thus the same chemical change  $k_1 I(a-x)^m$  will produce less visual effect when multiplied by a smaller  $\alpha$ , which is why light appears dimmer under diminished oxygen.

Let us now add light to a part of the visual field to which the eye is adapted. The intensity which needs to be added before its presence is recognized as extra brightness is  $\Delta I$ . The increase in photochemical change produced by this added light  $\Delta I$  is  $dx/dt = k_1 \Delta I (a-x)^m$ ; but since we are interested in the final visual effect we must include the conversion factor  $\alpha$  and consider

$$di/dt = \alpha k_1 \Delta I (a-x)^m \quad (4)$$

as describing the burst of impulses produced by the added light and photochemical change.

Suppose that we recognize an increase in brightness when the number of impulses leaving the retina (or reaching the cortex) is increased by a constant amount  $c$ . This makes equation (4) equal to  $c$  and yields

$$\Delta I = \frac{c}{\alpha k_1 (\alpha - x)^m} \quad (5)$$

on solving for  $\Delta I$ . Similarly in (3a) one can solve for  $I$  and get

$$I = \frac{k_2 x^n}{\alpha k_1 (\alpha - x)^m} \quad (6)$$

and as a result derive  $\Delta I/I$  by dividing (5) by (6). This yields

$$\Delta I/I = c / k_2 x^n \quad (7)$$

from which  $x$  can be eliminated only in terms of equation (3a) when  $m$  and  $n$  are given specific values. Previous work (Hecht, 1937) has shown that for cone vision both  $m$  and  $n$  equal 2. As a result, equation (3a) yields

$$x = \frac{a}{1 + 1/(\alpha K I)^{1/2}} \quad (8)$$

where  $K = k_1/k_2$  for convenience. If we now substitute this value of  $x$  from (8) into (7) we get

$$\Delta I/I = C \left[ 1 + \frac{1}{(\alpha K I)^{1/2}} \right]^2 \quad (9)$$

where  $C = c/a^2 k_2$ .

Under constant physiological conditions the conversion factor  $\alpha$  is constant and cannot be separated from the combined constant  $K$  which is merely the ratio  $k_1/k_2$ . Therefore the relation between  $\Delta I/I$  and the brightness  $I$  should be described by equation (9) with or without  $\alpha$ ; and as a matter of history, that equation does fit all brightness discrimination data from the earliest by Aubert (1865) to the most recent by McFarland, Halperin, and Niven (1944).

When equation (9) is plotted on a double logarithmic grid as in Fig. 4 the shape of its curve is constant and independent of the parameters  $C$ , and  $\alpha K$ . These merely locate the position of the curve on the axes:  $C$  on the ordinates and  $\alpha K$  on the abscissas. The measurements in Fig. 4 as well as those of McFarland, Halperin, and Niven show that oxygen lack does not change the shape of the curve, nor does it change its position on the ordinates. This means that  $C$  remains unchanged. But oxygen lack does displace the curve to higher intensities, which means that either  $\alpha$  or  $K$  is altered.

The constant  $C$  stands for  $c/a^2 k_2$ , and since  $C$  is not altered by oxygen lack, its components are also not altered, unless we make the extremely unlikely assumption that the change in the numerator is balanced by a precisely similar change in the denominator. The data, however, say that either  $\alpha$  or  $K$  or both

are altered, and become smaller the lower the oxygen concentration. The constant  $K$  equals  $k_1/k_2$ , and we have just seen that  $k_2$  is unaltered. It is also very unlikely that  $k_1$  is altered by oxygen lack, because  $k_1$  has the dimensions of light absorption (Hecht, 1924) and does not change with temperature or oxygen (Hecht, 1921; Bruner and Kleinau, 1936; Chase and Hagan, 1943). Therefore  $K$  is probably uninfluenced by lack of oxygen, and leaves the conversion factor  $\alpha$  as the only parameter to be changed under the circumstances.

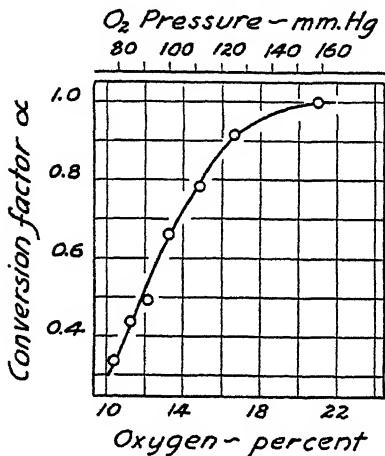


FIG. 5

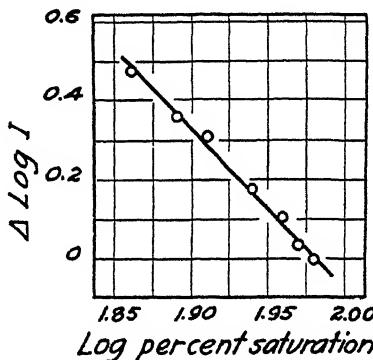


FIG. 6

FIG. 5. Relation between oxygen concentration and the conversion factor  $\alpha$  which translates chemical change into physiological magnitude. The value of  $\alpha$  is assumed unity at sea level.

FIG. 6. Relation between percentage saturation of the blood and the effect on brightness discrimination. The value  $\Delta \log I$  is the distance which the brightness discrimination function in Fig. 4 has to be displaced along the  $\log I$  axis for different degrees of anoxia. The slope of the straight line is 4.

This seems sensible because the conversion probably involves the action of bipolar and ganglion cells which are sensitive to oxygen lack.

From the measurements one can find precisely how  $\alpha$  varies with oxygen concentration. Table IV gives the horizontal displacement along the  $\log I$  axis which the curves suffer at different oxygen concentrations. According to equation (9) a constant  $\Delta I/I$  requires  $\alpha K I$  to be constant. Since oxygen lack shifts the curve, and thus increases  $I$ , this means that  $\alpha$  varies inversely with the intensity increment. If we assume that  $\alpha$  is 1 at sea level, we can compute its value for any other oxygen concentration from the numbers in Table IV by remembering that  $\log \alpha$  will be 0 minus the increase in  $\log I$  for that oxygen concentration.

The computed values of  $\alpha$  are shown in Fig. 5 against the oxygen concentration. The relation between the two seems reasonable; the curve is steep at low oxygen concentrations and flattens off at normal oxygen values of air at sea level. The shape of the curve is reminiscent of the upper part of the oxygen saturation curve of blood. Its vertical extent, however, is much greater than the saturation curve for the same oxygen concentrations.

There is actually a simple relation between  $\alpha$  (or its equivalent, the shift in light intensity) and the arterial saturation at different oxygen concentrations. From Chart B3 of the Handbook of respiratory data in aviation<sup>2</sup> we have taken the average arterial saturation corresponding to the oxygen concentrations used in our experiments. In Fig. 6 these arterial saturations are plotted as abscissas against the intensity shift of the  $\Delta I/I$  curve, both on a log scale. The straight line through the points has a slope of 4, which means that the log  $I$  shift or its equivalent  $\alpha$  varies as the fourth power of the arterial saturation. Considerably more knowledge about oxygen action will be necessary before such a fourth power relation can have meaning but its presence indicates that relatively slight changes in oxygen saturation produce large changes in vision.<sup>3</sup>

## VII

### SUMMARY

1. Brightness discrimination has been studied with individuals breathing oxygen concentrations corresponding to 7 altitudes between sea level and 17,000 feet. The brightnesses were 0.1, 0.01, and 0.001 millilambert involving only daylight (cone) vision.

2. At these light intensities, brightness discrimination begins to deteriorate at fairly low altitudes. The deterioration is obvious at 8,000 feet, and becomes marked at 15,000 feet, where at low brightness, the contrast must be increased 100 per cent over the sea level value before it can be recognized.

3. The impairment of brightness discrimination with increase in altitude is greater at higher altitudes than at lower. The impairment starts slowly and becomes increasingly rapid the higher the altitude.

4. Impairment of brightness discrimination varies inversely with the light intensity. It is most evident under the lowest light intensities studied, but

<sup>2</sup> Published by the Office of Scientific Research and Development.

<sup>3</sup> Added in proof. The essentials of these ideas were communicated by letter to Dr. R. A. McFarland and have been so reported in a recent paper by McFarland, Halperin, and Niven on Visual thresholds as an index of physiological imbalance during insulin hypoglycemia (*Am. J. Physiol.*, 1946, **145**, 299). Apparently insulin hypoglycemia affects brightness discrimination much as does anoxia, and our formulation of the basis for the shift of the  $\Delta I/I$  curve along the log  $I$  axis holds for this as well as for anoxia.

shows in all of them. However, it decreases in such a way that the deterioration is negligible in full daylight and sunlight.

5. The thresholds of night (rod) vision and day (cone) vision are equally affected by anoxia.

6. The quantitative form of the relation between brightness discrimination  $\Delta I/I$  and the prevailing brightness  $I$  remains the same at all oxygen concentrations. The curve merely shifts along the log  $I$  axis, and the extent of the shift indicates the visual deterioration.

7. The data are described in terms of retinal chemistry. Since anoxia causes only a shift in log  $I$  it is shown that the photochemical receptor system cannot be affected. Instead the conversion of photochemical change into visual function is impaired in such a way that the conversion factor varies as the fourth power of the arterial oxygen saturation.

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# THE POTENTIOMETRIC ANALYSIS OF MEMBRANE STRUCTURE AND ITS APPLICATION TO LIVING ANIMAL MEMBRANES

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In 1911 Loeb and Beutner concluded from experiments on the concentration potentials of the apple-skin that it is specifically permeable for cations. Since then electromotive forces produced by living membranes and organs in contact with salt solutions have often been used in analysing living membranes. In particular Osterhout has succeeded in elucidating the fine structure of the membrane of large plant cells by potentiometric analysis, while Höber (1926) and others investigated the ion permeability of animal membranes. In spite of such important results, it is not yet quite clear, whether the cation or anion selectivity which can be deduced directly from the measured potential is due to the sieve structure of the membrane or its charge or its specific dissolving power, or to all these factors taken together. A more detailed analysis of these properties will therefore be possible only if it is based on quantitative relations between the measured potential and those properties of the membrane or of the living tissue which influence the passage of ions. In order to make a survey of all these factors it seems reasonable to visualize first of all the molecular structure of living membranes.

Living membranes, or any other living matter, can be considered as a network of flexible primary valence chains, in particular protein chains (K. H. Meyer, 1928, 1929; Seifriz, 1928). These chains are interlinked at definite places by secondary valences or cross-linkings, called by us "regions of contact" (*Hafstellen*) and later by Frey-Wyssling (1938) "points of contact" (*Hafipunkte*). Intracellular fluid, ions, lipoids, and soluble globular proteins are located inside this network. The lipoids are mostly linked to apolar groups of the protein chains, for which the term lipophilic groups was proposed, while the hydrophilic groups of the chains are surrounded by water or aqueous cellular liquid and the ionised groups, e.g.  $-\text{COO}^-$  groups, are neutralised by mobile "counter-ions."

A natural membrane can moreover consist of layers of different composition under which lipoid layers can exist, and these layers can in turn be formed of areas of different properties like a mosaic.

## *Calculation of the Membrane Potential*

If a membrane, i.e. a layer of any kind separates two solutions of a binary electrolyte, an electromotive force  $E$  results from diffusion of the two solutions

through the layer. According to the Nernst equation its value is determined by the ratio of the transport numbers  $\frac{n_K}{n_K + n_A} : \frac{n_A}{n_K + n_A}$  which is equal to the ratio  $n_K/n_A$ :  $n_K$  being the number of cations and  $n_A$  the number of anions passing through the membrane per time unit.

$$\frac{dE}{dc} = \frac{RT}{F} \frac{n_K - n_A}{n_K + n_A} \frac{1}{c} \quad (1)$$

The ratio  $n_K/n_A$ , and therefore the value of  $E$  depends upon several factors.

1. *The ratio  $U_K/U_A$  of "migration velocities" or "mobilities" of cations ( $U_K$ ) and anions ( $U_A$ ) through the membrane.* A network with narrow pores allows the passage of small ions and holds up the larger ions ("sieve effect").  $U_K/U_A$  assumes thus a different value than it has in water. In a membrane, the pores of which are filled with a non-aqueous liquid, e.g. a lipoid, the ratio,  $U_K/U_A$  is also different from its value in water.

2. *Membrane Selectivity.* A network of chain molecules with laterally affixed ionised  $-\text{COO}^-$  groups neutralised by mobile cations ("counter-ions") is permeable for cations. For the cations of the solution outside can change place with the mobile cations (counter-ions) inside the membrane and thus permeate.

A membrane with fixed ionised basic groups, (e.g.  $-\text{NH}_3^+$ ), neutralised by mobile ions is permeable for anions. A membrane consisting of amphoteric molecules, e.g. protein chains, is not selective at the isoelectric point; it is permeable for cations on the alkaline side, for anions on the acid side of this point.

The concentration of fixed ions expressed in equivalents per liter with respect to the liquid in the pores is a characteristic constant for a membrane, called below "selectivity constant" and indicated by  $A'$  (K. H. Meyer and coworkers, 1936, 1945).

3. *The Concentration of Electrolytes.* While in a membrane permeable for cations at a low exterior concentration only the cations permeate and carry electricity, at greater concentrations an increasing amount of neutral electrolyte penetrates into the pores, so that anions also participate in carrying electricity. If the migration velocity of these anions is greater than that of the cations, more anions can pass through the membrane in the time unit than cations, so that beyond a certain concentration the electromotive force changes sign. (Teorell, 1935; Meyer, 1935; Meyer and Sievers, 1936). This phenomenon has long been known as "concentration effect" and nearly always erroneously attributed to a "reversal" of the membrane selectivity.

4. *The Solubility of Ions in the Membrane.* If the pores are wide and filled with water, it can be assumed that ions possess the same solubility in the pore liquid as in water. In membranes with narrow pores however, the attracting or repelling forces of the groups on the pore surface can no longer be neglected.

Increase or decrease of salt concentration in the pore liquid may follow. The salt will thus seem either more or less soluble than in the exterior aqueous phase. Moreover membranes can also be filled with a liquid other than water in which ions have a different solubility. We can take this into consideration by introducing the partition coefficients  $l_K$  (cation) and  $l_A$  (anion), which are equal to concentration in the pore liquid divided by concentration in water, or to activity coefficient in water divided by the activity coefficient in the pore liquid.

To begin with it is best to make the following simplifying assumptions.

1. The membrane is homogeneous; *i.e.*, it does not consist of layers of different properties and is not a mosaic.
  2. The concentration of the electrolytes in the liquid layers which are in direct contact with the membrane surfaces is constant, so that an unvarying flow of ions is established within the membrane (and a stationary state on both surfaces).
  3. The partition coefficients are independent of the concentration of the salt.
- We then arrive at the following equation:

$$E = \frac{RT}{F} \left[ u \cdot \ln \frac{x_2/A + u}{x_1/A + u} + \frac{1}{2} \cdot \ln \frac{(x_1/A + 1)(x_2/A - 1)}{(x_1/A - 1)(x_2/A + 1)} \right] \quad (2)$$

where  $u = \frac{U_K - U_A}{U_K + U_A}; \quad x = \sqrt{4x^2 + A^2}; \quad A = \frac{A'}{\sqrt{l_K \cdot l_A}}$

### *The Membrane Potential in the Study of Membranes*

By measuring the membrane potential at various concentrations of the electrolyte one obtains several equations from which the unknown quantities

$$U_K/U_A \text{ and } A = \frac{A'}{\sqrt{l_K \cdot l_A}}$$

can be deduced.

By comparing the values of  $A$  for the same membrane and salts with the same anion one obtains:

$$A_1 : A_2 = \frac{A'}{\sqrt{l_{K_1} \cdot l_A}} : \frac{A'}{\sqrt{l_{K_2} \cdot l_A}} = \sqrt{\frac{l_{K_2}}{l_{K_1}}}$$

The absolute values of  $A'$  and of  $l_K$  and  $l_A$  cannot be determined. One relates these values to  $l_{K^+}$  and  $l_{Cl^-}$  taken as units.  $A$ , determined with KCl, is thus by definition equal to  $A'$ , the "selectivity constant."

Similarly the migration velocities  $U_K$  and  $U_A$  are related to  $U_{Cl^-}$  taken as unity. The ratio  $U_{K_1}/U_A$  to  $U_{K_2}/U_A$  for two salts with the same anion gives the value for  $U_{K_1}/U_{K_2}$ . It is thus possible to relate migration velocities of all cations to that of  $K^+$  and this to that of  $Cl^-$ .

Serious objections to this theory have been recently formulated by Sollner (1944). He compared the values of  $A_p$  (concentration of fixed ions in the pore liquid determined by the potentiometric method) with  $A_b$  (the concentration of fixed ions determined by base exchange capacity and the water content of the membrane). He finds large discrepancies between  $A_p$  and  $A_b$ , which he attributes to some "inherent weakness" of the theory.

We wish to emphasize in the first place that according to our theory one cannot determine by potentiometric analysis the absolute value of  $A_p$ , but only the value of  $\frac{A'}{\sqrt{l_K \cdot l_A}}$ . Secondly, Sollner neglects, in deriving the value of  $A_b$ , the well known Donnan hydrolysis of insoluble acids. His simplified Equation 3 is thus erroneous. In the third place, the experimental method used to analyse the base exchange capacity is open to severe criticism. The fact that the presence or absence of salt does not alter in any appreciable manner the pH reading, proves that the observed variations of pH are within the limits of experimental error. Indeed if cation absorption is very small, considerable errors may arise from the solubility of glass or impurities in the solutions or the water. We think therefore that Sollner's results do not in any way invalidate our theory.

#### *Determination of A and $U_K/U_A$ by Means of Graphs*

In order to simplify the evaluation of the potentials one can use a system of graphs based upon the following principles. As shown in Equation 2 the value of  $E$  depends on  $u$  (*i.e.* on  $U_K/U_A$ ), on  $x_1/A$ , and  $x_2/A$  (*i.e.* on  $c_1/A$  and on  $c_2/A$ ). If the ratio of concentrations is kept constant, *e.g.*  $c_1:c_2 = 1:2$ , then  $E$  depends on two parameters only, namely  $U_K/U_A$  and  $c_1/A$ . For a given value of  $U_K/U_A$  (*e.g.*  $U_K/U_A = 1$ ) a curve is drawn of  $E$  as a function of  $\log A/c_1$ . For every other value of  $U_K/U_A$ , one obtains another curve (Fig. 1). This graph serves as a basis for the evaluation.

A series of measurements of  $E$  are now made keeping the ratio of concentration of electrolytes equal to 1:2, *i.e.* 0.01/0.02 N; 0.02/0.04 N; 0.04/0.08 N; 0.08/0.16 N; 0.16/0.32 N. These readings of  $E$  are entered in the graph as functions  $\log 1/c_1$ . We then obtain a curve, the "selectivity curve," which is characteristic for the membrane and the electrolytes chosen.

The experimental curve is now compared with the curves of the graph. We ascertain which curve it can be made to fit by displacement parallel to the abscissae. This gives the value of  $U_K/U_A$ . We then measure the displacement: it is equal to the difference  $\log A/c_1 - \log 1/c_1$  and therefore equal to  $\log A$ . As already mentioned the real "selectivity constant"  $A'$  cannot be determined by this method, but only the value  $A = \frac{A'}{\sqrt{l_K \cdot l_A}}$ . Here  $A$  measured with KCl is the "selectivity constant" of the membrane.

#### *Selective Permeability for H Ions*

Where several electrolytes are involved, all cations participate in the transport of positive electricity and contribute thus, according to their concentra-

tion, partition coefficient, and migration velocity to  $U_K$ . The same occurs with the anions.

If a Na phosphate buffer solution of pH 6 diffuses directly in a similar solution of pH 7, there results practically no electromotive force as the action of H ions, in spite of their greater velocity, is extremely small with respect to the

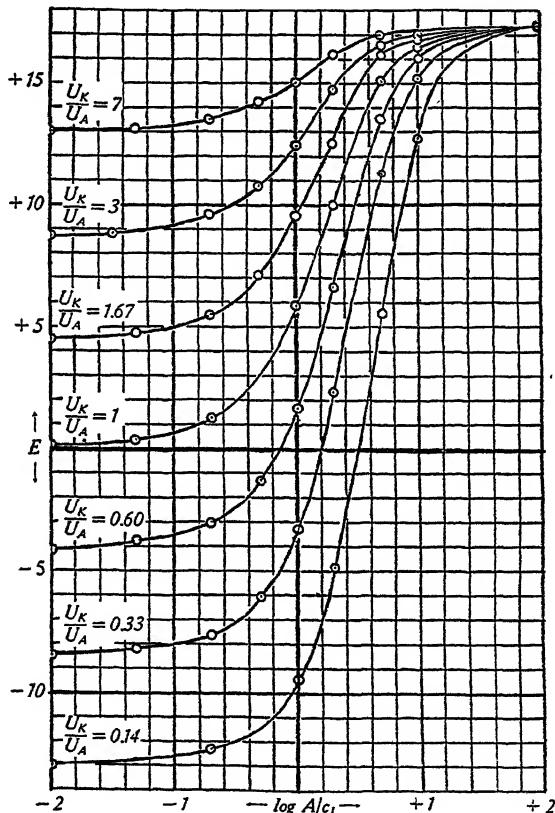


FIG. 1. Potential difference  $E$  as a function of  $\log A/c_1$  for different values of  $U_K/U_A$ .

influence of Na ions, the concentration of which is  $10^5$  times greater. If, however, a membrane permeable only to H ions and impermeable to all other ions is set between the two solutions, there results a membrane potential, such as would be the case, if only the H ions were present, the others being excluded from carrying electricity and therefore from influencing the membrane potential. The existence of such specific H ion permeability is thus best revealed by means of buffer solutions.

One cannot, however, distinguish whether a membrane is permeable for H ions

only or OH ions, or both. One can only say that a membrane is impermeable to all ions except H or OH. For, as the product  $[H^+][OH^-]$  remains constant, the effect of the H ions in the two solutions equals that of the OH ions:

$$E = \frac{RT}{F} \ln \frac{[H^+]_1}{[H^+]_2} = \frac{RT}{F} \ln \frac{[OH^-]_2}{[OH^-]_1} = \frac{RT}{F} 2.30(pH_2 - pH_1) \quad (3)$$

at 15° one obtains:  $E = 57 (pH_2 - pH_1)$  mv. For greater simplicity, we shall speak of this as H ion permeability.

A membrane of this type is the well known glass membrane of the "glass electrode." Many synthetic organic membranes, e.g. the collodion membrane of Michaelis (1926) exhibit the phenomenon of specific H ion selectivity in a very imperfect way: the migration velocity of all other ions is only greatly diminished with respect to the velocity of H ions, but is not reduced to zero.

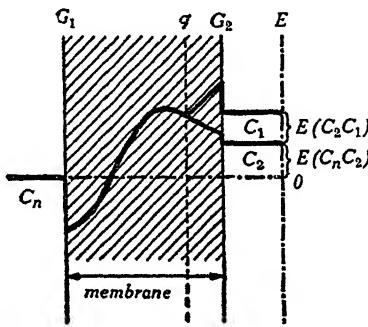


FIG. 2. Analysis of outer layers of membrane.

#### *Analysis of a Membrane Consisting of Layers*

In the case of membranes consisting of layers of different permeability, the total potential observed can be considered as the sum of the individual potentials of each single layer. But only the outer layers can be analysed in the following way. The concentration of the solution on one side of the membrane is kept constant, while on the other side solutions with increasing concentrations are successively brought to bear, each concentration doubling the previous one; i.e.,  $c_1, c_2 = 2 c_1, c_3 = 2 c_2$ , etc.  $E$  is measured immediately after the change of the solution. Only in the outer layer  $q - G_2$  (Fig. 2) is equilibrium immediately obtained, while between  $G_1$  and  $q$  the preceding ion distribution remains at first unchanged, so that the sum of individual potentials is kept constant. The potential  $c_1$ /outer layer/ $c_2$  is given by the difference  $E(c_n/c_2) - E(c_n/c_1)$ . Thus by plotting this number and the following:  $E(c_n/c_3) - E(c_n/c_2)$ , etc., the selectivity curve can be drawn. The value  $A$  and the ratio  $U_K/U_A$  for the boundary layer can then be obtained as described above.

H ion selectivity can be tested by putting buffer solutions of different pH

successively in contact with one surface of the membrane and measuring the difference between the potentials obtained.

If the pores of the outer layer are wide, the adjacent solution soon penetrates towards the inner layers. Hence the electromotive force is obviously influenced and may slowly change.

If, after a while, the electromotive force reaches a constant level, it can be assumed that the solution has reached a deeper narrow pored layer through which diffusion is very slow.

If a membrane in layers is bathed by the same solution on either side, no potential will be observed if equilibrium is attained. If this is not the case the membrane presents the phenomenon of "*asymmetric potential*." This can be explained in the following way. If the ions of the inner layers of the membrane, on their way into the adjacent solutions or *vice versa* from the solutions into the interior have to pass through layers of different selectivity on both sides of the membrane, a concentration potential will arise. This asymmetric potential disappears more or less rapidly with death, while living processes maintain the disequilibrium.

If a membrane is of the "mosaic type," its permeability and selectivity are determined by the areas of greatest permeability.

#### *Application of the Method to the Analysis of the Frog Skin*

A great number of synthetic membranes have been analysed according to the principles explained above. We shall now undertake to apply this method to a living membrane, namely the skin of a frog's belly. As the membrane is in layers, only the relevant methods can be employed. A general remark must, however, be made. The electromotive force obtained, after having changed the solution on one side of a living organ, often does not remain constant, but changes slowly. This variation might be caused by permeation through an inner layer as mentioned above. But in dealing with living membranes, an entirely different phenomenon may occur; *i.e.*, excitation can provoke biological reactions which involve a changing of ionic concentration or permeability inside the living membrane. Thus the true cause of variation cannot always be determined.

#### *Experimental Method, Material, and Treatment*

Experiments were carried out on healthy, well fed specimens of frogs (*Rana temporaria*) caught in May or September. The frogs are beheaded, the skin of the belly is cut out, and stretched with needles on a flat cork ring *K* of 32 mm. inner diameter. The skin *H* is then firmly placed in the apparatus shown in Fig. 3 between two rubber stoppers *C* with cylindrical holes of 10 mm. diameter. The two rubber stoppers are pressed against each other by means of three screws *S*, through two parallel brass plates *P* with holes, so that the frog skin separates the two inner chambers in the rubber stoppers. Through these holes two glass vessels *A* and *B* are brought to within 7

mm. of the membrane. In cell *A* a solution of constant concentration flows slowly along the membrane without interruption during the whole series of readings. In cell *B* too the solution flows slowly over the membrane; this solution can be rapidly changed. The apparatus is slightly inclined so that tube *R*, slightly curved down-

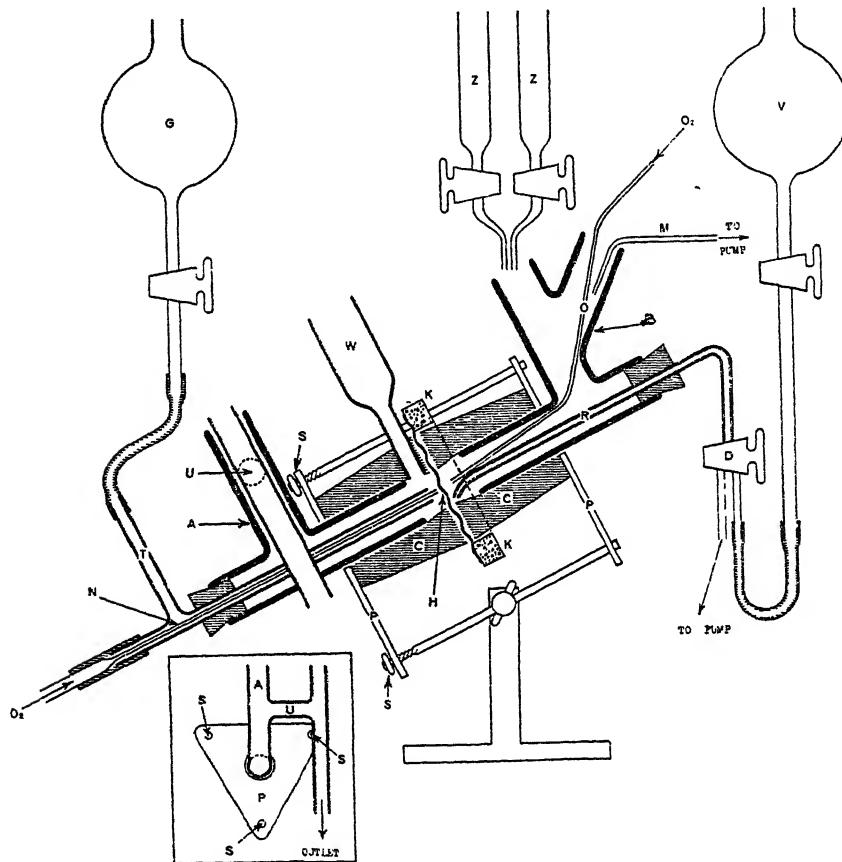


FIG. 3. Apparatus for the examination of frog skin.

wards at its end and brought as near as possible to the membrane has its orifice at the lowest point of cell *B*. Through this tube the solution  $L_1$  of cell *B* can be drawn away in a few seconds by means of a water pump. The new solution  $L_2$  can then be turned on from one of the reservoirs *Z*, with a wide tap. By again drawing off the solution through tube *R* and refilling with solution  $L_2$ , cell *B* is rapidly and thoroughly washed and finally filled with solution  $L_2$  which flows in slowly from a reservoir *V* through tube *R* after turning the three-way tap *D*. Excess of solution is drawn away by the water pump through a capillary tube *M*. Oxygen bubbles through the solution through another capillary tube *O* reaching close to the membrane. Cell *A* is fitted

with a T-tube  $T$  one end of which goes close to the membrane. Through the other end protruding above, a solution is let in from reservoir  $G$ , and the excess flows away through tube  $U$ . Through the third branch of tube  $T$  a capillary tube  $N$  is set close to the membrane to allow bubbling of oxygen. The gas escapes through the water level tube  $W$ . Cells  $A$  and  $B$  are connected by means of strings soaked with dilute KCl solution with vessels containing saturated KCl solution; the latter are connected to calomel electrodes. Readings are carried out by means of a compensation bridge. As zero instrument an electrometer triode is used, the grid-circuit of which is linked to the compensation bridge. A galvanometer is placed in the anode circuit. By

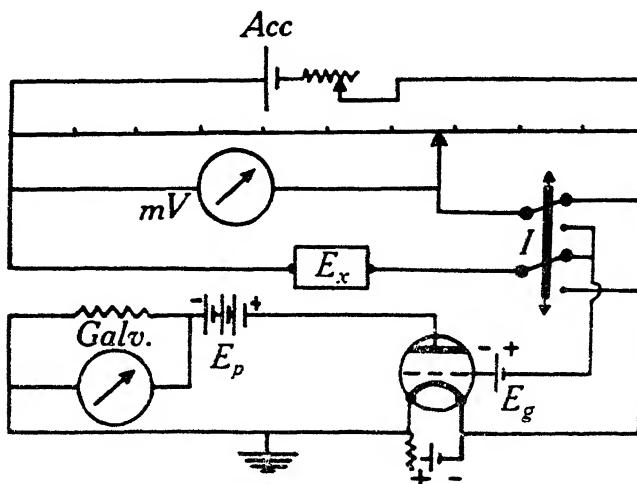


FIG. 4.  $E$ , membrane with unpolarisable electrodes;  $I$ , double switch;  $E_a$ , grid polariser;  $E_p$ , anode battery.

means of a double-pole switch in the grid-circuit it can be observed—individually of the slow variations of anodic current—whether the potential is exactly compensated or not. The sensibility of the bridge is about  $\pm 0.5$  mv. (*cf.* Fig. 4).

In order to study the outer epithelial layer of the skin, the inner side is brought in contact with Ringer solution (6.5 gm. NaCl, 0.2 gm. KCl, 0.2 gm. CaCl<sub>2</sub>, 0.32 gm. NaHCO<sub>3</sub>, 1 gm. glucose made up to 1 liter with water). The pH of 7.7 to 7.8 is checked with a quinhydrone electrode. The outer side of the skin is brought into contact with solutions of NaCl or KCl, beginning with the concentration of 0.01 N, then this is doubled and so on.

In order to study the inner layer of connective tissue (corium), the epithelial side is brought into contact with tap water. Water in Geneva contains about 50 mg. Ca and 12 mg. Mg per liter as hydrogen carbonates, chlorides, and sulfates, pH = 8.

To determine the specific H ion permeability, the following buffer solutions—practically isotonic with Ringer solution—were used.

Solution of	2N NaCl	0.1N $\text{Na}_2\text{HPO}_4$	0.1N HCl	Filled to
	cc.	cc.	cc.	cc.
pH 7.7-7.8	50	50	2	1000
pH 7.4	50	50	5.5	1000
pH 6.8-7.0	50	50	19	1000
pH 6.5	50	50	28.5	1000

In these solutions the concentration of  $\text{Na}^+$  and phosphate ions remains constant, that of  $\text{Cl}^-$  ions varies not more than from 0.1000 to 0.1030, and hydrogen ion concentration alone increases to twenty times its value. The pH values of the solutions are checked by means of the quinhydrone electrode. They vary slightly with the water used and the age of the solution, due to variations of  $\text{CO}_2$  content.

In several experiments Ringer solution with which  $\text{CO}_2$  had been mixed, was used as acid solution. Its pH was each time potentiometrically determined (pH 6.8 to 6.0).

#### *The Influence of Respiration*

When the skin of a freshly killed frog is bathed on both sides by Ringer solution through which oxygen bubbles to maintain respiration, the liquid touching the inner surface (corium, connective tissue) is charged positively. The potential rises gradually and after an hour at most it reaches a practically constant level of 50 to 100 mv. If, however, the skin is bathed by Ringer solution on the inside and tap water on the outside, the potential immediately obtained is of a lower value and sometimes even of opposite sign. It changes progressively in the same manner as stated above; *i.e.*, the inner surface (corium) becomes more positive till a practically constant level is reached after about an hour. This potential is, however, from 30 to 60 mv. less positive than in the first case. Reactions which slow down combustion processes in the cell, *e.g.* deprivation of oxygen or cooling, cause the resting potential to become progressively more negative with respect to the inner surface. After letting in oxygen or after heating to room temperature it regains its former value. Hydrocyanic acid which interrupts all combustion processes in the cell, makes the inner surface rapidly more negative. If then Ringer solution is applied on both sides, the potential may differ considerably from zero; thus the HCN does not lead to a destruction of cell membranes which would cause the potential to sink to zero.

All these observations are in good agreement with the important statement made by several previous authors that the resting potential depends upon respiration. As Mansfeld (1910) and later Leuthardt and Zeller (1934)

pointed out, lack of oxygen renders the potential on the inner side (corium) more negative. It is shifted in the same direction by all influences such as removal of glucose which slacken the combustion process. Huf (1935) showed that the potential falls in spite of the presence of glucose, when the skin has been poisoned with bromoacetic acid and can therefore no longer oxidize glucose. On adding lactate which can be utilized even by poisoned cells, the potential rises and returns to normal.

The influence of temperature studied in detail by Barnes (1939) is most typical. Cooling down to 4–5° C. causes a drop of potential in the course of 15 to 30 minutes. Further cooling to 0° produces another slow decrease which finally ceases. Heating to 20° causes the potential to rise very rapidly to normal. The increase can amount to considerably more than 50 mv. The relative rise of the potential is thus many times greater than the relative rise of  $T$ . There is in fact no proportionality between  $T$  and the potential observed, as should be the case if the effect of temperature on the potential was merely a physical one. According to Barnes, inhibition and acceleration of combustion processes caused by variation of temperature, make the potential fall and rise.

Narcotics such as chloroform or fatty alcohols (Leuthardt and Zeller) and poisoning by deuterium oxide (Barnes) also lead to a fall in potential; if the narcotics or the deuterium oxide are washed away, the potential returns to its normal value.

Before beginning experiments with salt solutions, one must wait till a practically constant resting potential is attained. Temperature and oxygen supply must be kept constant throughout the experiment in order to exclude all possible variations of potential due to influence of respiration.

Sometimes the resting potential fails to become entirely constant, but decreases slowly and regularly. This is probably due to a certain slowing down of combustion processes in the living cells. However, the change of potential generated by varying the solutions is so rapid that this slow decrease does not interfere with the readings.

If the potential takes time to stabilize, for instance if buffer solutions are applied to the inner surface, the double influences of metabolism and buffer solution are superposed. Each value can, however, be evaluated from the curves obtained (*viz.* Fig. 8, curve 6, page 370).

The skin remains alive in the apparatus about 12 hours. The living condition can be tested by cutting off the oxygen; this ought to cause a drop of potential if the skin is alive.

#### *Study of the Outer Surface of Frog Skin*

When the outer epithelial surface is bathed in tap water and the inner one (corium) in Ringer solution, a low negative potential with respect to the outer

surface is generally produced. By substituting distilled water for tap water, the electromotive force changes sign. This can be explained if tap water ions penetrate into the outer layer and pass out again through a membrane which

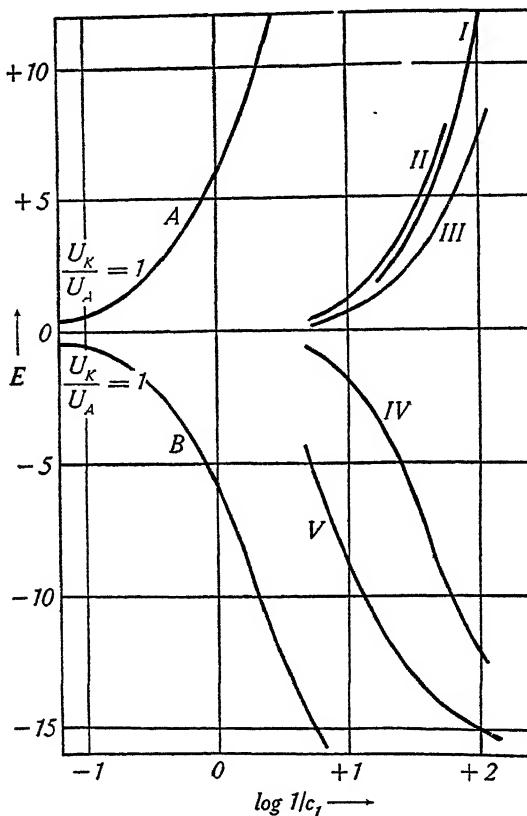


FIG. 5. Selectivity curves of frog skin. I, NaCl without buffer; II, treatment with Ringer, then KCl buffered to pH 7.8; III, KCl after treatment with  $\text{CHCl}_3$ ; IV, KCl buffered to pH 7.8 without preliminary treatment; V, KCl without buffer, without preliminary treatment; A, reference curve for cation selectivity; B, reference curve for anion selectivity.

is chiefly permeable for cations, thus imparting to the adjacent solution a positive charge. The outer epithelial layer is thus normally permeable for cations.

For a further study of the outer layer, Ringer solution was applied inside and salt solutions outside, beginning with the concentration of 0.01 N, then 0.02, 0.04, 0.08, etc. The salt solutions were rendered isotonic with Ringer

by addition of sucrose. By means of the differences of the potentials measured, the selectivity curve was drawn (Fig. 5 and the following table). When, at the end of each series of readings, the first solution was applied again, the first potential was restored immediately or very rapidly.

Curve I, Fig. 5

Interior		Exterior		E	Differences relative to exterior		
				mv.		mv.	
+	Ringer	0.01 N NaCl	—	-62			
+	Ringer	0.02 N NaCl	—	-74	+	0.01/0.02	—
+	Ringer	0.04 N NaCl	—	-80	+	0.02/0.04	—
+	Ringer	0.08 N NaCl	—	-83	+	0.04/0.08	—

For curve 1, Fig. 5, A was found equal to 0.02, the membrane being cation-permeable. All other frogs gave similar values.

The selectivity curves for KCl are quite different. If the skin is bathed by Ringer inside and KCl outside, no constant potential is attained and the potential differs with each specimen tried. This can only be caused by an active reaction of the skin.

If on the other hand KCl solution is applied on both sides of the skin, constant and reproducible potentials are observed. But as is shown by the following table and the selectivity curve V (Fig. 5), the exterior epithelial membrane has become permeable for anions.

Curve V, Fig. 5

	Interior		Exterior		E	Differences relative to exterior		
					mv.		mv.	
(Beginning)	+	0.16 N KCl	0.16 N KCl	—	-1			
	+	0.16 N KCl	0.01 N KCl	—	-52.5			
	+	0.16 N KCl	0.02 N KCl	—	-37.5	—	0.01/0.02	+
	+	0.16 N KCl	0.04 N KCl	—	-23.5	—	0.02/0.04	+
	+	0.16 N KCl	0.08 N KCl	—	-11	—	0.04/0.08	+
	+	0.16 N KCl	0.16 N KCl	—	-1	—	0.08/0.16	+
	-	0.16 N KCl	0.32 N KCl	+	+5	—	0.16/0.32	+
(End)	+	0.16 N KCl	0.16 N KCl	—	-1			

The membrane has thus changed from being cation-permeable and acid in character to anion-permeable and basic. The epithelial surface of the skin must therefore be amphoteric and consist of amphoteric proteins. Amberson (1936) found this in dead frog skin. Its isoelectric point is given by him as pH 5.1. On its alkaline side it is permeable to cations, on the acid side to anions.

This anion permeability which occurs under the influence of K ions, can only be explained by production of acid under the action of potassium which brings the intercellular fluid to the acid side of the isoelectric point. This explanation

accounts also for the curious and varying results obtained by placing Ringer against KCl: the alkalinity of Ringer more or less neutralises the newly formed acid.

Distinct cation selectivity, however, can be obtained also with KCl, if the skin is placed for a while before the experiment in Ringer or 0.1 N NaCl buffered with phosphate to pH 7.8 and if the KCl at pH 7.8 is applied outside (curve II, Fig. 5). That the acidifying process must be considered as an active reaction of the skin, can be shown by treating the skin beforehand with chloroform vapor: it then retains its normal cation permeability in the presence of KCl without the alkaline treatment (*cf.* curve III, Fig. 5).

The selectivity curves show that the selectivity constants for cation permeability and therefore the concentration of fixed acid groups is considerably lower ( $A \cong 0.02$ ) than when we have anion permeability ( $A = 0.05$  to 0.1). It can be inferred that either the proteins of the epithelium possess more bound basic than acid groups, or that the water content of the membrane is reduced in an acid milieu.

The ratio  $U_K/U_A$  is found to be near unity for NaCl as well as for KCl, while in water  $U_{Na}/U_{Cl}$  equals 0.6. The migration velocity  $Na^+$  in the membrane is thus relatively higher than in water, and practically as high as that of  $K^+$ .

Solubilities of  $K^+$  and  $Na^+$  in the epithelial layer cannot differ very much since the selectivity constants  $\frac{A'}{\sqrt{l_{K^+} + l_{Cl^-}}}$  and  $\frac{A'}{\sqrt{l_{Na^+} + l_{Cl^-}}}$  are practically the same.

The influence of different H ion concentrations on the potentials was studied by means of buffers from pH 6 to 8. There was no potential difference between the different buffers. The epidermis is thus not specifically permeable for H ions; it behaves quite differently from the inner surface of the skin as we shall see below.

#### *Study of the Inner Surface of Frog Belly Skin*

The curves of selectivity were determined with tap water on the epithelial side of the skin and with NaCl or KCl solutions on the inner surface. Within the limits of error, no potential difference was generated upon changing the concentration of the salts. The selectivity curves are straight lines parallel to the abscissae. Hence  $A = 0$ : there is no cation or anion selectivity.

In order to investigate the membrane with respect to H ion selectivity, tap water was applied to the outer surface and buffer as described above of pH 7.8 to the inner surface (corium). When the potential had remained constant for half an hour which was generally the case after 1 hour, a more acid solution, e.g. of pH 6.8, was applied. After a period of latency lasting several minutes, often even a quarter of an hour, the potential dropped at first slowly, then rapidly, the inner surface becoming more negative, till a potential difference

was obtained which corresponds almost exactly to exclusive H ion permeability, according to the equation (mv. at 15°).

$$E = \frac{RT}{F} \ln \frac{[\text{H}^+]_1}{[\text{H}^+]_2}; \quad E = \frac{RT}{F} 2.30(\text{pH}_2 - \text{pH}_1); \quad E = 57(\text{pH}_2 - \text{pH}_1)$$

When the first solution of pH 7.8 is again applied,  $E$  rises after a short period

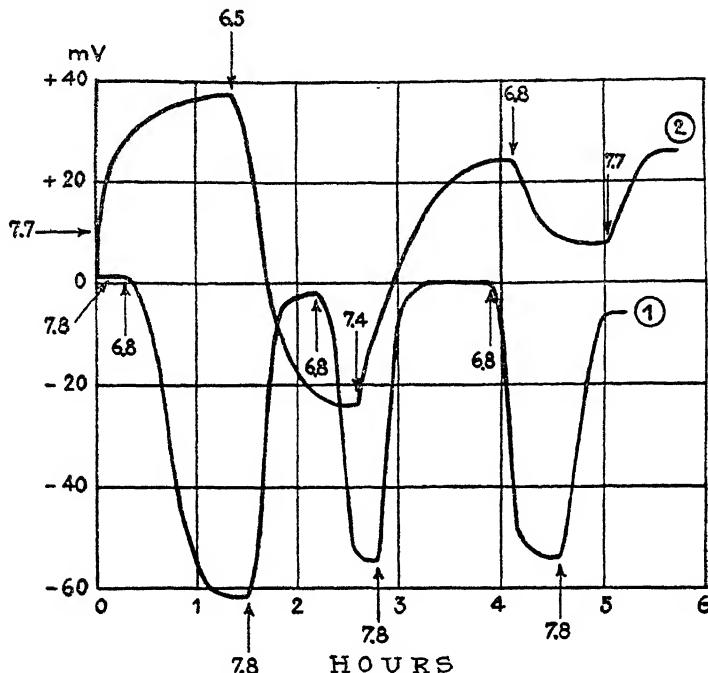


FIG. 6. Effect of phosphate buffer of different pH on the potential of frog skin. Buffer applied to corium, tap water to epidermis. Sign related to corium.

of latency to its former value. The whole cycle can be repeated several times (Figs. 6 and 7). The following measurements were read *inter alia*:

*Curve I, Fig. 6*

pH changes	$E$ observed mv.	$E$ calculated mv.
7.8/6.8	-63	-57
6.8/7.8	+60	+57
7.8/6.8	-53	-57
6.8/7.8	+55	+57
7.8/6.8	-54	-57
6.8/7.8	+48	+57

In the case of large, thick-skinned, September frogs, the potential falls and rises more slowly. We assume that this latent period is due to a porous, non-selective layer through which the buffer solution must pass before reaching the membrane selective for H ions further inside.

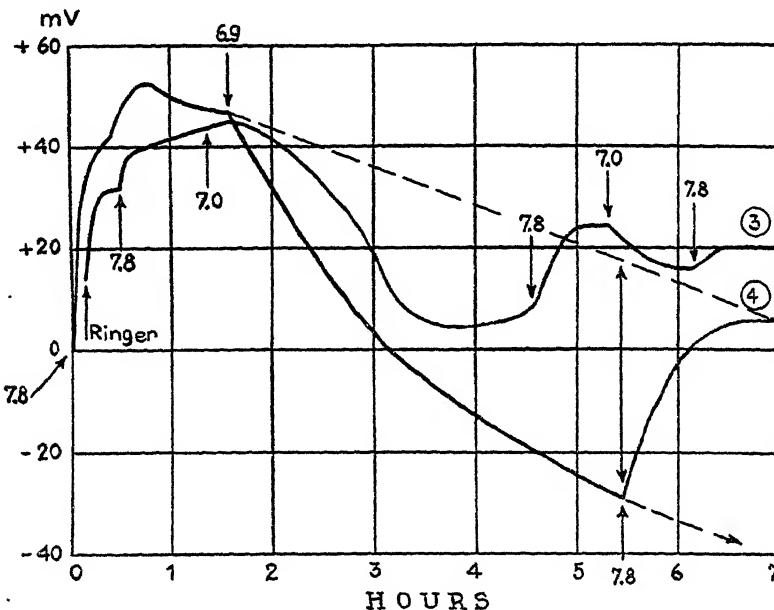


FIG. 7. Effect of phosphate buffer of different pH on the potential of frog skin. Buffer applied to corium, tap water to epidermis. Sign related to corium.

The membrane of these frogs soon loses its selectivity as shown; e.g. in curve 2, Fig. 6, and curve 3, Fig. 7.

pH changes	<i>E</i> observed	<i>E</i> calculated	
7.7/6.5	-61	-68	Curve 2, Fig. 6
6.5/7.4	+48	+51	
7.4/6.8	-16	-34	
6.8/7.7	+18	+51	
7.8/7.0	-41	-46	Curve 3, Fig. 7
7.0/7.8	+17	+46	
7.8/7.0	-8	-46	
7.0/7.8	+5	+46	

In some cases the resting potential did not remain quite constant; the gradual linear decrease seems due to a progressive slackening of combustion processes. If this descending straight line serves as starting point for calculations such as

in curve 4, Fig. 7, the following values are obtained for that part of the potential (indicated by a dotted line) for which the solutions are solely responsible.

Curve 4, Fig. 7

pH changes	<i>E</i> observed mv.	<i>E</i> calculated mv.
7.8/6.9	-46	-51
6.9/7.8	+46	+51

In order to shorten as much as possible the latent period caused by the slow penetration of the electrolyte through the porous layer, Ringer brought to an acid pH by CO<sub>2</sub> was used instead of the phosphate buffer. Its pH was determined potentiometrically each time. As this solution shows a considerable CO<sub>2</sub> tension, it might be supposed that CO<sub>2</sub> would rapidly penetrate through the porous layer of connective tissue and thus make the intercellular fluid which is in contact with the selective membrane inside, rapidly more acid. In fact, the fall and rise of potential followed almost immediately after the change of solution (Fig. 8 and following table).

pH changes	<i>E</i> observed mv.	<i>E</i> calculated mv.	
7.7/6.9	-47	-46	Curve 5, Fig. 8
6.9/7.7	+48	+46	
7.7/6.9	-37	-46	
6.9/7.7	+23	+46	
7.9/6.0	-53	-108	Curve 6, Fig. 8
6.0/7.9	+51	+108	
7.9/6.0	-46	-108	
6.0/7.9	+44	+108	
7.9/6.0	-51	-108	

In one experiment hydrocyanic acid in a 0.5 per cent solution was applied to the outer side and replaced after 10 minutes by tap water. This caused a considerable fall of potential due to the inhibition of respiration. But in spite of hydrocyanic acid poisoning, substituting at the inner surface pH 7.9 for pH 6, caused the potential to rise, while return to pH 6 made it fall, in exactly the same manner as with a normally breathing skin (curve 6, Fig. 8).

Curve 6, Fig. 8

pH changes	<i>E</i> observed mv.	<i>E</i> calculated mv.
6.0/6.0 (HCN)	-36	-
6.0/7.9	+74	+108
7.9/6.0	-47	-108

This confirms the hypothesis that the observed variations of potential are not due to a variation in respiration which is suppressed by hydrocyanic acid, but are caused by a direct physical-chemical action of the solution containing CO<sub>2</sub> on the H<sup>+</sup> selective membrane.

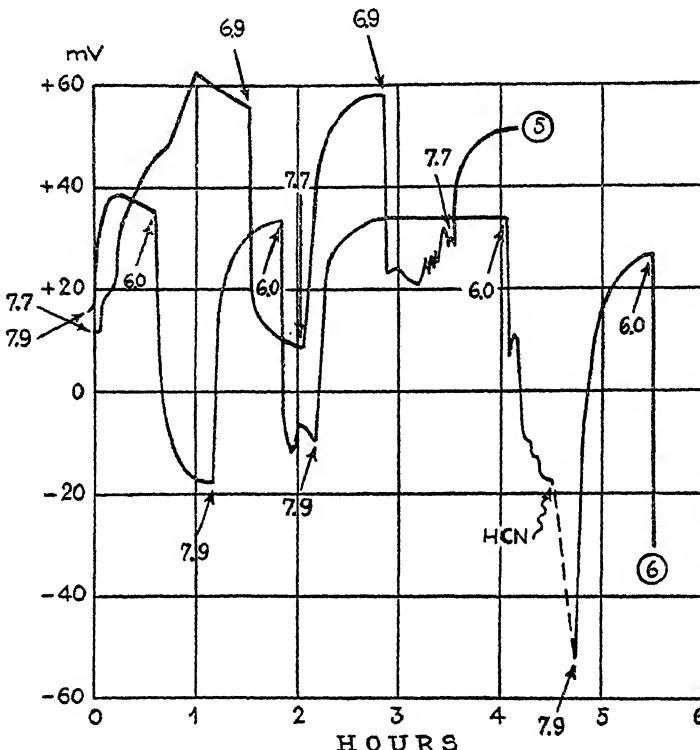


FIG. 8. Effect of Ringer + CO<sub>2</sub> of different pH on the potential of frog skin. Ringer applied to corium, tap water to epidermis. Sign related to corium.

## *Conclusions Concerning Skin Structure*

These experiments combined with known data lead to the following conclusions concerning the multilayered structure of the skin. The interior surface consists of a non-selective layer which is porous and easily permeable for large ions. Then comes a membrane which is practically exclusively permeable for H ions. This layer is very thin as the electrical resistance of the entire skin is rather low. Immediately adjoining this there is a layer in which carbonic acid is generated by respiration and passes in part into the serum and is then carried away. The acid diffuses out as  $\text{CO}_2$ . This involves a greater concentration of  $\text{CO}_2$  and therefore of  $\text{H}^+$  in the  $\text{CO}_2$ -producing layer than in the inter-cellular liquid on the other side of the thin selective membrane. The potential

difference caused by the different concentrations of  $H^+$  on both sides of the thin membrane depends obviously on the rate of production of  $CO_2$  and therefore on the intensity of the respiration. Next to the  $CO_2$ -producing layer, there are cells which under the influence of KCl can produce acid. Finally comes a layer of amphoteric proteins which form the outer surface.

We shall compare this picture built upon potentiometric measurements with histological data. Frog skin, 0.1 to 0.2 mm. thick, consists of an inner layer of connective tissue about half as thick as the whole skin and containing several "chromatophores" (layer 5, Fig. 9). This layer corresponds to the inner porous layer mentioned above. Next follows a very thin homogeneous

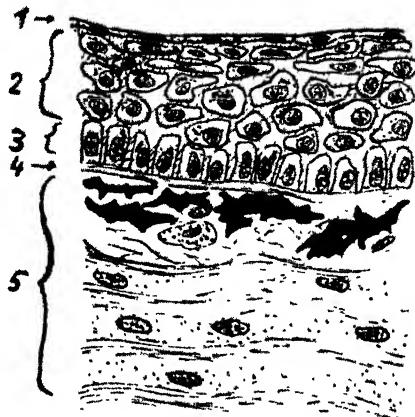


FIG. 9. Cross-section of frog skin. (1) Stratum squamosum; (2) epithelium; (3) stratum germinativum; (4) membrana basilaris; (5) corium.

membrane (4, Fig. 9), called basal membrane, which is obviously identical with the  $H^+$  selective layer. It stains with methyl red and must therefore consist of proteins of the collagen type. Close against the basal membrane is located the stratum germinativum, a layer of prismatic epithelial cells about  $7\mu$  thick and 10 to  $20\mu$  long (3, Fig. 9). These prisms are perpendicular to the skin surface. The cells are connected by protoplasmic fibres and constantly dividing to form other epithelial cells. These cells must have an intense metabolism and are probably the main source of carbonic acid. Then follow 5 to 7 layers of epithelial cells of equal size which are alive but do not divide. Finally at the outer surface there is a layer of flat dead epithelial cells (stratum squamosum). There is thus a large measure of agreement between our own conclusions and the histological picture of skin structure.

As has been pointed out, variation of the pH at the outer surface had no effect on the skin potential; only the variation at the corium side led to the

detection of the membrane permeable to H ions. When the corium was thick, the potential followed only slowly the pH variation. There can be little doubt that permeability to H ions of the basal membrane will be perfectly hidden, if the corium is very thick and tight. The absence of any effect on the potential, when the pH of the adjacent solution is changed, therefore does not prove the absence of an interior layer permeable to H<sup>+</sup> in a multilayered membrane or organ.

### *Conclusions Concerning the Resting Potential of Frog Skin*

As pointed out above the corium is separated from the living cells of the stratum germinativum by the thin basal membrane which is specifically permeable to H<sup>+</sup> ions. Carbonic acid is continuously produced in respiring cells; the CO<sub>2</sub> which diffuses out through the basal membrane is carried away by the serum and blood. At constant temperature and oxygen supply a stationary state will thus arise which involves a gradient of CO<sub>2</sub> and therefore of H ions on both sides of the basal membrane. Owing to this gradient a potential must exist across the membrane, the corium side being positive. This potential probably accounts for a great part of the asymmetrical potential which is observed when the skin is bathed in Ringer on both sides. It seems possible, however, that other ions produced in respiration processes contribute to the observed potential.

Any variation of the respiration and the CO<sub>2</sub> production of the living cells must influence the gradient of CO<sub>2</sub> and therefore the potential across the membrane. The very marked influence of respiration processes on the potential is thus explained.

If, on the other hand, the skin is bathed in Ringer inside and tap water outside, the observed potential is near zero. The potential across the membrane must, therefore, be compensated by a potential of opposite polarity which is located between the respiring cells and the tap water. If the fluid and the water are separated from each other by a layer which is permeable to cations, as is indeed the case (stratum squamosum), a potential must occur which is of opposite polarity to the potential across the basal membrane and can therefore compensate it.

### *Previous Work on the Ion Permeability of Skin*

(a) *Frog's Skin.*—Leuthardt and Zeller (1934) studied the ion permeability of frog skin by means of concentration potentials. They found the outer layer to be selectively permeable for cations even in the presence of KCl. Polyvalent electrolytes in medium concentration and univalent electrolytes in high concentration lead to a "reversal of the membrane selectivity." The observed reversal of polarity which occurs when passing from a lower to a higher concentration cannot be attributed to a reversal of the selectivity, but should be explained as follows: If the migration velocity of the anions is much greater than that

of the cations as in the case of  $\text{Cl}^-$  and slow polyvalent cations, then beyond a certain concentration more anions pass through than cations, and the electro-motive force changes sign.

(b) *Human Skin*.—The ion permeability of human skin was studied in particular by Rein (1926). Two fingers of the same hand are placed in two vessels containing KCl solution. The skin of one finger is injured, while the other is left intact. The solutions are connected in the usual manner to unpolarisable electrodes by means of concentrated KCl solution.

The concentration of the solution bathing the injured finger is kept constant ( $1 \text{ N KCl}$ ), while the other is increased from  $0.001 \text{ N}$  to  $0.01$ ,  $0.1$ , and  $1 \text{ N}$ . The differences between successive readings can be used in plotting the selectivity curve; the results of five series of experiments are given in the following table.

KCl						Average
	mv.	mv.	mv.	mv.	mv.	mv.
+	0.001/0.01	-	9	10	9	9.4
+	0.01 /0.1	-	4.2	11	3	9.6
-	0.1 /1.0	+	-4.2	-3	-5	-4
						+5
						-2

These potential differences may be recalculated for a concentration ratio  $1:2$ . The following values are thus obtained:

KCl	mv.	
+	0.003/0.006 -	+3
+	0.03 /0.06 -	+2
-	0.3 /0.6 +	-1

The outer surface of the human skin is thus found to be always cation-permeable, though the selectivity is very small ( $A = 0.001$ ) as is evident from the curve of selectivity which was drawn with the values of the last table. In addition a small influence upon  $U_K/U_A$  is observed. For high concentrations  $U_K/U_A$  approaches the value 0.8 instead of 1 as in water. The reversal of potential beyond a certain concentration is not to be attributed to a reversal of skin permeability, but to the increasing influence of rapidly migrating anions as pointed out above.

Rein's experiments with  $\text{AlCl}_3$  can be explained in the same manner. The following values were obtained by subtracting the potentials measured in four series of experiments:

AlCl <sub>3</sub>					Average	
	mv.	mv.	mv.	mv.	mv.	
+	0.0001/0.001	-	+40	+28	+21	+25
-	0.001 /0.01	+	-9	-8	-1	-3
-	0.01 /0.1	+	-18	-27	-27	-23
-	0.1 /1.0	+	-20	-24	-20	-28.5
						-24

In very dilute solution the cation  $\text{Al}^{+++}$  alone carries electricity across cation-permeable membranes: however a concentration of 0.001 N is already sufficient to provoke a reversal of polarity due to penetration of  $\text{Cl}^-$  ions which migrate much more rapidly than  $\text{Al}^{+++}$ .

The conclusion that human skin is cation-permeable, is in agreement with the disturbances of neutrality observed by Rein and known as the "Bethe-Toropoff effect" (1914, 1915). If a direct current flows through a cation-selective membrane, a column of anions migrates towards the anode and a similar column of cations towards the cathode. But the anions are held back by the membrane and accumulate on the cathodic side, thus attracting H ions through the membrane from the anodic side. Thus an acid reaction is produced on the cathodic and an alkaline reaction on the anodic side. Under the anode the interior fluid of the skin becomes acid while under the cathode it becomes alkaline. The pain felt by the person subjected to the experiment was probably due to a change of pH in the intercellular fluid of the skin tissue.

#### *Study of the Muscle Fibre with Respect to H Ion Selectivity*

The fact that membranes with selective H ion permeability exist in living organs induced us to investigate the membrane of muscle fibre. For such experiments the buffer solutions ought to be applied directly to the membrane sheathing a single fibre, while the other solution should be applied to the interior of the fibre or to an injured spot. But as we cannot manipulate isolated fibres in our laboratory, we had to carry out the experiment with whole muscles. It is, however, practically impossible to bring a solution containing large ions, such as phosphate ions into direct contact with the majority of the fibres inside since diffusion through the interstitial fluid is much too slow. The potential thus observed will be principally a diffusion potential between buffer and interstitial fluid, while the membrane potential will not be modified. Assuming that  $\text{CO}_2$  would penetrate more rapidly inside the interstitial fluid and modify the pH in the immediate vicinity of at least a great part of the fibres, we exposed the uninjured part of the muscle alternately to air and a mixture of 80 per cent  $\text{CO}_2$  and 20 per cent oxygen. One electrode was connected by means of Ringer or Ringer +  $\text{CO}_2$  with this uninjured part, the other electrode with the injured part which was kept constantly in oxygen or air. The full magnitude of the potential difference for exclusive H ion selectivity can, however, by no means be expected because of the slow diffusion and shunting through the interstitial fluid.

The muscle (*sartorius* of *temporaria*) is cut off at the pelvis, keeping the insertion at the knee intact. It is then placed in a chamber with two compartments separated from each other by a partition with a hole in it. The muscle is pulled through the hole until one half of the muscle is in each compartment. The hole is then stopped

up with vaseline. In this manner the uninjured and injured halves of the muscle can be exposed to different gases. Strings soaked in Ringer are laid on the muscle to establish contact with the electrodes.

The compartments were first filled with air until the injury potential remained constant (20 to 30 mv., the uninjured side being positive). Then the CO<sub>2</sub> mixture was let into the compartment containing the uninjured part. As soon as a constant value was obtained, air was brought back again. The

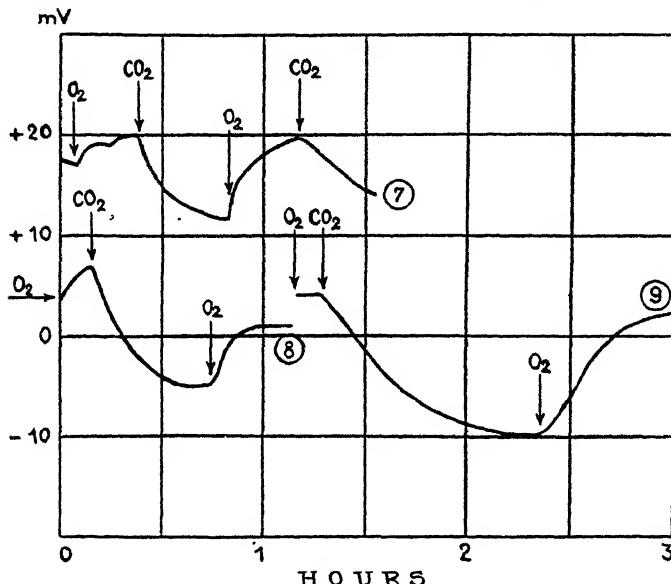


FIG. 10. Effect of a mixture of 80 per cent CO<sub>2</sub> + 20 per cent O<sub>2</sub> (curve labelled CO<sub>2</sub>) and air (curve labelled O<sub>2</sub>) on the potential of frog sartorius. Curve 7, injured muscle; CO<sub>2</sub> applied to the non-injured part; the intact half in O<sub>2</sub>. Curves 8 and 9, intact muscle.

carbonic acid caused in all experiments a fall in potential of 6 to 7 mv. on the uninjured side. When CO<sub>2</sub> was replaced by air (O<sub>2</sub> in the graph) the potential rose to its former value (e.g. curve 7, Fig. 10).

During the experiments the muscle lost its excitability. But even the unexcitable muscle continued to respond to carbonic acid and its potential rose again when carbonic acid was replaced by air.

Further experiments were carried on with uninjured muscles. The potential observed when both halves of the muscle were in air, was rather small. Introduction of CO<sub>2</sub> into one compartment brought the potential here to the negative side (curves 8 and 9, Fig. 10) and air made it rise again.

These results seem to be quite clear: the muscle membrane or sarcolemma is

specifically permeable to H ions. The sarcolemma shares this property with the basal membrane; it is furthermore stained by the same dyes as the latter and consists therefore of similar proteins.

This statement is not in agreement with the widely accepted view that the muscle membrane is selectively permeable only for K ions. We think, however, that the experimental evidence for this latter theory is by no means conclusive. One of the arguments is the swelling of normal muscle in KCl solution isotonic to physiological NaCl. It seems to prove that potassium permeates through the membrane while sodium does not. But as Verzár (1945) showed recently, muscles when poisoned with iodoacetic acid swell much more slowly in KCl. The swelling after application of KCl seems thus the consequence of a complicated biological response of the muscle and not a simple osmotic phenomenon.

As Buchthal and Lindhard (1936) found, the injury potential of an isolated muscle fibre increased from 27 to 52 mv., when the temperature rose by 12.5°. The potential dropped in the course of 8 minutes by cooling, while on heating the former value was restored in less than 1 minute. The behaviour of the muscle is thus very similar to that of the skin as described by Barnes (1939). This statement in connection with our observation on the selectivity for H ions of the sarcolemma leads us to the following conception of the injury potential of muscle: the concentration of metabolic products, especially carbonic acid, is greater within the respiring fibre than in the interstitial fluid from which the CO<sub>2</sub> is constantly diffusing out into the blood. Owing to the gradient of CO<sub>2</sub> and to the greater concentration of HCO<sub>3</sub><sup>-</sup> ions in the serum a concentration difference of H ions will be established on both sides of the sarcolemma. A potential difference will thus arise across this membrane, the interstitial fluid being positive, the sarcoplasm negative. Since at the injured spot only a diffusion potential of small magnitude can arise (at the junction of the adjacent liquid and the sarcoplasm), we must assume that the major part of the observed injury potential is due to the membrane potential across the sarcolemma. Any variation of the pH inside the sarcoplasm must thus cause a variation of the resting potential; alkalinization must express itself by a shift of the potential in the negative, and acidification in the positive direction. It follows that the shift in the negative direction which is observed during the action current very probably indicates an alkalinization inside the fibre.

#### *The Permeability of the Nerve Membrane*

The selectivity constant of the external layer of frog sciatic can be deduced from concentration potentials measured by van Heuverswyn (1936).

A N/10 KCl solution was applied to two different spots of a nerve which had remained previously in isotonic glucose solution. An asymmetric potential between +6 and -6 mv. was observed. If one electrode remained unchanged and the 0.1 N

KCl solution on the other was replaced by 0.01 N KCl, the potential rose 20 mv. higher at the latter electrode. As can be calculated, a concentration ratio of 2:1 would cause a change of potential of about +6 to +8 mv. The outer layer is thus permeable for cations. If we admit for the ratio  $U_K/U_A$  the same value as in water, i.e. 1:1, the selectivity constant is found to be  $A = 0.02$  to  $0.03$ . If 0.1 N NaCl and 0.001 N are successively applied, a potential near zero, as indeed was found by van Heuwerswyn can be expected, if the ratio  $U_{Na}/U_{Cl}$  remains the same as in water; i.e., equal to 0.6. This is due to the fact that the cation selectivity is compensated by a greater migration velocity of the Cl ions.

A similar study was undertaken by van Heuwerswyn (1938) on the non-medullated nerve of lobster. After replacing 0.1 N NaCl by 0.001 N a potential difference of 77 mv. was produced which corresponds to a value of 11.5 mv. for a concentration ratio of 2:1. The selectivity constant must therefore be about  $A = 0.03$ . The outer layer is thus somewhat more selective for cations than in frog sciatic.

We are very much indebted to Dr. A. van der Wyk and Dr. Anderegg for their valuable advice and help.

#### SUMMARY

1. The electromotive forces which arise, if two electrolyte solutions are separated from each other by a layer of any kind, are discussed. A general equation is derived comprising the known equations for diffusion, partition, and membrane (Donnan) potentials as special cases.
2. A method is proposed to analyse membranes potentiometrically with respect to their cation or anion selectivity, their dissolving power for ions, and their influence on ion mobility (migration velocity).
3. The possibility of analysing a membrane composed of several layers of different permeability is discussed.
4. The investigation of the skin of the belly of *Rana temporaria* leads to the following results. It is composed of at least four layers of different permeability, one of which is specifically permeable to H ions and is very likely identical with the "basal membrane" situated between the stratum germinativum and the corium. The major part of the resting potential of the skin is located across this membrane and is due to the difference of  $H^+$  concentrations on both sides of the membrane.
5. Experiments on muscle show that the sarcolemma is specifically permeable to H ions. The injury potential of the muscle is attributed to the difference of  $H^+$  concentration inside and outside the fibre.

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## ISOLATION OF HEXOKINASE FROM BAKER'S YEAST\*

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Hexokinase was first described by Meyerhof (2), who prepared the enzyme from baker's yeast and demonstrated its stimulatory effect on the fermentation of hexoses by muscle extracts. Through the experiments of von Euler and Adler (3), and Meyerhof (4), it was established that this enzyme catalyzes a transfer of phosphate from adenosinetriphosphate (ATP) to hexoses, thus initiating the fermentation or oxidation of the latter. Colowick and Kalckar (5) showed that only the terminal phosphate group of ATP was transferred to hexose, and demonstrated that the primary products of the reaction were adenosinediphosphate (ADP) and hexose-6-phosphate. The reaction catalyzed by yeast hexokinase may thus be written as follows:—



In the present paper a method is described for the isolation of hexokinase in highly purified form from baker's yeast. Glucose, fructose, and mannose are all acted upon by the enzyme, but the rate of phosphorylation varies with the sugar used. The turnover number, when the phosphate acceptor is glucose, is 13,000 moles of substrate per  $10^6$  gm. of protein per minute at 30° and pH 7.5 and is not changed by repeated crystallization of the enzyme. The specific activity is approximately 30 times that of the crude yeast extract, indicating that hexokinase constitutes about 3 per cent of the extracted protein.

### *Measurement of Hexokinase Activity*

The manometric method described by Colowick and Kalckar (5) was adopted. The method is based on the fact that one acid equivalent is liberated per mole of phosphate transferred from ATP to glucose.

A Warburg vessel, the side bulb of which contained:

0.2 cc. 0.04 M ATP<sup>1</sup> (sodium salt, pH 7.5)

\* This work was carried out under National Defense Research Committee contract No. OEMsr-123, under the auspices of the Office of Scientific Research and Development, which assumes no responsibility for the statements contained herein. A preliminary report has already appeared (1).

<sup>1</sup> The rate of the hexokinase reaction depends to a considerable extent on the degree of purity of the ATP sample used. When a commercial sample of ATP was used, the

0.05 cc. 0.1 M NaHCO<sub>3</sub>, 0.2 M MgCl<sub>2</sub>

and the main compartment of which contained:

0.5 cc. 0.054 M NaHCO<sub>3</sub>

0.1 cc. 0.5 M glucose

0.75 cc. hexokinase solution

was filled with 5 per cent CO<sub>2</sub>-95 per cent N<sub>2</sub> (pH 7.5 at 30°). The amount of protein used varied from 100 γ for crude extracts to as little as 2 γ for the pure enzyme. After equilibration for 10 minutes at 30°, the stopcock was closed and the contents of the side bulb and main compartment were mixed. CO<sub>2</sub> evolution in the absence of glucose (due to adenylylpyrophosphatase action) was negligible except with crude hexokinase preparations, in which cases it was necessary to apply a small correction.

The validity of the manometric method was checked by comparing the CO<sub>2</sub> evolution with the amount of phosphate transferred from ATP to glucose. Samples were incubated, without and with glucose, and fixed with trichloro-

TABLE I  
*Comparison of CO<sub>2</sub> Liberated with P Transferred to Glucose in Hexokinase Test System*

Experiment No.	P transferred to glucose	CO <sub>2</sub> liberated
	micromoles	micromoles
1	4.77	4.70
2	4.60	4.70
3	4.65	5.00
4	5.20	5.30
5	2.45	2.36
6	2.97	3.10

acetic acid at the end of the manometric measurement. The filtrates were analyzed for the acid-labile P of ATP by determining orthophosphate (6) before and after 11 minute hydrolysis in N H<sub>2</sub>SO<sub>4</sub> at 100°. The difference in labile P in the two samples was a measure of the amount of phosphate transferred from ATP to glucose, since the hexose-6-phosphate formed is not appreciably hydrolyzed under the above conditions. From Table I it can be seen that 1 mole of CO<sub>2</sub> was evolved in the manometric test per mole of phosphate transferred.

*Determination of Specific Activity.*—One unit of hexokinase is defined as the amount of enzyme causing an initial rate of CO<sub>2</sub> evolution of 1 c.mm. per minute under the specified condition of the test. The initial rate was readily determined by measuring CO<sub>2</sub> evolution during the first 8 minutes, since,

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rate was only about 65 per cent of that observed with samples prepared in this laboratory. Values for specific activity recorded in this paper were obtained with ATP which had been carefully purified and which did not contain an appreciable amount of ADP.

within certain limits of enzyme concentration, the rate was always linear during this period.

The level of purity of a given enzyme preparation is expressed in terms of the specific acitivity; *i.e.*, the number of units per mg. of protein. Protein was determined colorimetrically by means of the biuret reaction (7) after precipitation with trichloroacetic acid.

The degree of accuracy of the specific activity determination is illustrated by the data in Table II. The activity of a crude hexokinase preparation was tested over an eightfold range of protein concentration. The number of units of activity found was proportional to the amount of protein added, so that the specific activity (units per mg.) was independent of protein concentration, the average value found being 103 units per mg., with a maximum deviation from the mean of  $\pm 5$  units.

TABLE II  
*Proportionality between Enzyme Concentration and Activity in Hexokinase Test System*

Crude yeast hexokinase	$\text{CO}_2$ liberated (0-8 min.)	Specific activity
mg. protein per test	c.mm.	units per mg. protein
0.0109	9	103
0.0218	19	109
0.0436	34	98
0.0872	71	102

### Purification

The procedure used may be outlined briefly as follows:—

(1) Autolysis of yeast with toluol, extraction with water, and precipitation of extracted proteins with 50 per cent of alcohol, essentially as described by Meyerhof (2). (2) Removal of an isoelectric precipitate. (3) Two fractionations with alcohol at  $0^\circ$ , the material soluble in 25 to 29 per cent alcohol but precipitated by 45 to 48 per cent alcohol being collected. (4) Adsorption of the active material on  $\text{Al}(\text{OH})_3$  gel, and elution with phosphate buffer. (5) Fractionation with alcohol at  $0^\circ$ , the material soluble in 40 per cent alcohol but precipitated by 57 per cent alcohol being collected. (6) Fractionation of a more concentrated protein solution with alcohol at  $-7^\circ$ , resulting in removal of amorphous material with 20 per cent alcohol, removal of an as yet unidentified crystalline protein fraction with 24 per cent alcohol, and removal of more amorphous material with 29 per cent alcohol. (7) Crystallization from ammonium sulfate solution.

Several points in this outline deserve comment. In the early work, the extraction of the autolyzed yeast was carried out at  $35^\circ$ , the temperature recommended by Meyerhof (2). In later experiments the extraction was carried out

at 5°, as recommended by Kunitz and McDonald,<sup>2</sup> who found a greater yield of hexokinase at this extraction temperature.

It was found desirable to carry out the extraction and early fractionation procedures in the presence of glucose, since, as first reported by van Heyningen<sup>2</sup>, crude preparations of hexokinase lose activity rapidly on standing unless glucose is present. The protective action of glucose or fructose is illustrated in Fig. 1. Incubation of a partially purified hexokinase preparation (300 units per mg. protein) in  $\text{NaHCO}_3\text{-CO}_2$  buffer, pH 7.5, at 30° for 30 minutes resulted in the loss of

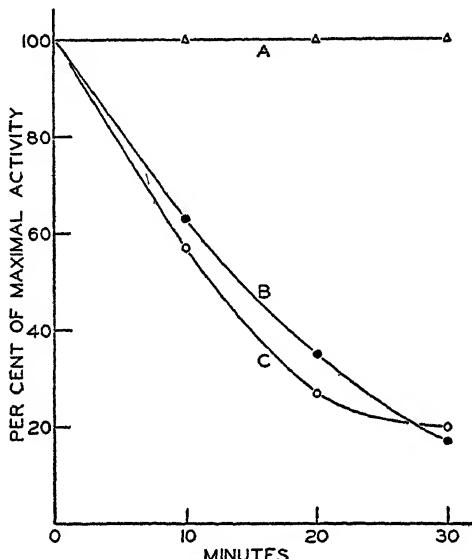


FIG. 1. Protective action of glucose or fructose on crude yeast hexokinase. 22 γ of protein per cc. (300 units per mg.) incubated at 30°, pH 7.5, and tested for hexokinase activity after 10, 20, and 30 minutes. Curve A shows the stability of the enzyme when either glucose or fructose (0.037 M) was present during the incubation period. Curves B and C show the loss in activity toward fructose and glucose, respectively, when these sugars were absent during the incubation period.

about 80 per cent of the activity, the loss of activity toward fructose and glucose occurring at about the same rate. The presence of glucose or fructose (0.037 M, the concentration used in the manometric system) completely prevented this inactivation. The concentration of glucose present during the various stages in the isolation procedure was about 1 per cent (0.055 M).

Fractional precipitation with alcohol was always carried out at acid reaction (pH 5.5) at 0° or below. Under these conditions, the total recovery of hexo-

<sup>2</sup> This information was made available to us through confidential reports circulated by the National Defense Research Committee.

kinase in the various fractions obtained from a given solution was practically quantitative. Attempts to fractionate with alcohol at pH 7 resulted in a complete loss of hexokinase activity.

Close temperature control was essential in carrying out fractional precipitation with alcohol. It will be noted that in some cases fractionation was carried out at 0°, in other cases at -5° to -7°. Slight variations in temperature in this range result in marked changes in protein solubility. For example, protein fractions completely soluble in 20 per cent alcohol at 0° could be largely precipitated by lowering the temperature to -5°.

Purification was achieved almost entirely by means of fractional precipitation with alcohol. The only other procedure used which resulted in marked purification was the adsorption of the enzyme on aluminum hydroxide. The chief

TABLE III  
*The Purification of Yeast Hexokinase*

Fraction	Total protein gm.	Total units	Recovery of units per cent	Specific activity
				units per mg. protein
Crude extract from 11 kg. yeast	40.50	4,050,000		100
1	29.40	2,646,000	65	90
2	21.50	2,365,000	58	110
3	2.93	998,000	25	340
3a	2.00	800,000	20	400
4	1.18	755,000	19	640
5	0.23	368,000	9	1600
6	0.060	167,000	4	2780
6a	0.043	129,000	3	3000
Crystals				3000

value of this procedure lies in the fact that a large amount of gummy material of non-protein nature remains unadsorbed and can be discarded. The enzyme activity can then be recovered quantitatively, and with a considerable increase in specific activity, by elution with phosphate buffer. It will be noted that, after this purification step, the concentration of alcohol required to precipitate the enzyme is considerably higher than that required in the earlier stages.

The following is a detailed protocol for one experiment in which 43 mg. of hexokinase were isolated from 40 gm. of yeast proteins, extracted from 11 kg. of yeast. The results are summarized in Table III.

*Preparation of Crude Extract.*—Eleven kg. of fresh, starch-free, Anheuser-Busch<sup>3</sup> baker's yeast were warmed from 15° to 39° over a period of 2 hours. 750 cc. of toluol

<sup>3</sup> We are grateful to the staff of the Anheuser-Busch yeast plant for their cooperation in providing the facilities necessary for the large scale preparation of the crude yeast extracts.

were added and thoroughly mixed with the yeast. After 45 minutes at 37-40°, the yeast had partially liquified. 500 cc. of 50 per cent glucose were added and the mixture was stirred for an additional 45 minutes. Eleven kg. of shaved ice and 250 cc. of 50 per cent glucose were added, and the mixture was kept at 5° for 18 hours.

The suspension was centrifuged at 5° in 250 cc. bottles. The slightly cloudy supernatant fluid (11.5 liters, pH 6.2) was stored at 0° overnight. This solution is designated "crude extract" in Table III.

1. *Precipitation with Alcohol*.—The protein of the crude extract was precipitated by the addition of an equal volume of cold 95 per cent ethanol. Care was taken to prevent the temperature from rising above 5° during the addition of the alcohol. The mixture was allowed to stand for 1 hour before centrifuging in the cold in 250 cc. bottles at 2000 r.p.m. The precipitate was suspended in 1 per cent glucose and mechanically stirred overnight at 5°. It was then centrifuged in the cold yielding about 2 liters of supernatant fluid (fraction 1).

2. *Isoelectric Precipitation of Inert Proteins*.—The pH of fraction 1 was adjusted at 0° to pH 4.7 by the slow addition of 75 cc. of M acetic acid. The precipitate which formed was centrifuged off in the cold, and the supernatant fluid (fraction 2) was adjusted to pH 5.6 by the addition of 30 cc. of 1.5 N NaOH. A flocculent precipitate formed but was not removed.

3. *Fractionation with Ethanol at 0°*.—The temperature was maintained at 0° while 900 cc. of ethanol were slowly added (final concentration of alcohol was 29 per cent). The resulting precipitate was discarded after centrifugation. The ethanol content of the supernatant fluid was increased to 45 per cent and the suspension was centrifuged in the cold. The syrupy precipitate was dissolved in 1 per cent glucose (410 cc., fraction 3).

Fraction 3 was diluted with 400 cc. of 0.1 M acetate buffer, pH 5.4, and 270 cc. of cold ethanol were slowly added (25 per cent final alcohol concentration). The precipitate obtained after centrifugation was discarded and the ethanol content of the supernatant fluid was increased to 48 per cent at 0°. The syrupy precipitate was centrifuged down and dissolved in 1 per cent glucose (200 cc., fraction 3a).

4. *Adsorption of Hexokinase on Al(OH)<sub>3</sub>*.—Fraction 3a was diluted with an equal volume of 0.02 M acetate buffer, pH 5.4, containing 1 per cent glucose. 160 cc. of Al(OH)<sub>3</sub> gel<sup>4</sup> were added to the solution at 0°. The suspension was centrifuged in the cold, the supernatant discarded, and the residue washed three times with 500 cc. portions of 0.01 M acetate buffer, pH 5.4, containing 1 per cent glucose. The washed residue was eluted three times with 200 cc. portions of cold 0.1 M phosphate buffer, pH 7.2. The eluates were combined (600 cc., fraction 4).

5. *Fractionation with Ethanol at 0°*.—Fraction 4 was adjusted to pH 5.3 by the addition of 42 cc. of M acetic acid, and cold ethanol was slowly added until the alcohol concentration was 40 per cent. The suspension was centrifuged and the precipitate was discarded. The ethanol concentration of the supernatant fluid was increased to 57 per cent and the precipitate obtained by centrifugation in the cold was dissolved in 1 per cent glucose. A small amount of insoluble material was removed by centrifugation and the pH of the supernatant was readjusted to pH 5.4 by the addition of acetate buffer (fraction 5, 150 cc.).

<sup>4</sup> Alumina C<sub>v</sub>, prepared as described by Willstätter (8).

6. *Fractionation with Ethanol at -7°.*—Fraction 5 was treated in the cold with 180 cc. of ethanol and the precipitate was suspended in 20 cc. of 0.01 M acetate buffer, pH 5.4, containing 1 per cent glucose. A small amount of insoluble material was removed by centrifugation.

The supernatant solution (23 cc.) was dialyzed in the cold for 2 hours against 2 liters of 0.01 M acetate buffer, pH 5.4, containing 1 per cent glucose. The dialyzed solution (41 cc.) was treated with 10 cc. of ethanol at 0°, cooled to -7°, and the precipitate was discarded. The alcohol content was increased to 24 per cent at -7°, and crystals (hexagonal plates) appeared within 2 days. After several more days, the crystalline material was removed by centrifugation at -5°. This crystalline protein fraction has not as yet been identified. 40 cc. of supernatant fluid were obtained (fraction 6).

The ethanol content of fraction 6 was raised to 29 per cent at 0° and the solution was cooled slowly to -5°. The material which precipitated was amorphous and was removed. The hexokinase in the supernatant fluid (fraction 6a) had a specific activity of 3000 units per mg. protein which could not be increased by further fractionation with alcohol or by crystallization from ammonium sulfate.

*Crystallization.*—Attempts to crystallize hexokinase from fraction 6a with ethanol under various conditions were uniformly unsuccessful. Crystallization from ammonium sulfate, under the conditions recommended by Kunitz and McDonald,<sup>2</sup> was readily achieved as follows:

The ethanol content of an aliquot of fraction 6a was raised to 60 per cent at -4° in order to recover the protein. The precipitate was dissolved in 0.1 M phosphate buffer, pH 7.0, and saturated ammonium sulfate was added to slight turbidity. The solution was kept at 3° and long needle-like crystals of hexokinase formed in a few days. A photograph of these crystals has already been published (1).

#### *Properties of the Crystalline Enzyme*

*Effect of Magnesium Ions.*—The crystalline enzyme is inactive in the absence of Mg ions. In Fig. 2, the activity is plotted against the MgCl<sub>2</sub> concentration. It can be seen that the concentration required to produce one-half of the maximal activating effect is  $2.6 \times 10^{-3}$  M (dissociation constant for the Mg-protein complex). The concentration used routinely in the manometric tests,  $6.5 \times 10^{-3}$  M, is sufficient for practically maximal activity.

Sodium fluoride, which inhibits the activity of a number of enzymes requiring Mg ions, had no effect on the activity of crystalline hexokinase when tested in concentrations ranging from 0.006 to 0.125 M. Warburg and Christian (9) showed that the inhibition of enolase by fluoride depended on the presence of orthophosphate. The inorganic phosphate concentration in the hexokinase test system was approximately 0.001 M. The effect of fluoride at higher phosphate concentrations has not been investigated.

*Action on Various Hexoses.*—Studies on substrate specificity showed that the crystalline enzyme acted on d-glucose, d-fructose, and d-mannose, the relative rates with the three sugars being 1:1.4:0.3. That a single protein is

responsible for the actions on glucose and fructose is indicated by the following observation. When the enzyme was tested in the presence of a mixture of glucose and fructose, each present in sufficient concentration to saturate the enzyme, the rate was intermediate between the rates observed with glucose and fructose alone. This result suggests a competition between glucose and fructose for the same enzyme.

*Effect of Proteins on Inactivation by Dilution.*—The activity of the crystalline enzyme, in contrast to the crude enzyme (Table II), was not proportional to enzyme concentration, the activity at low protein concentrations being considerably lower than that calculated from the dilution. This loss of activity

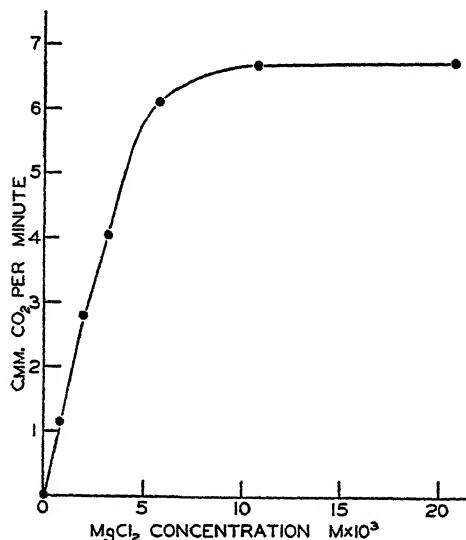


FIG. 2. Effect of Mg<sup>++</sup> concentration on the activity of crystalline yeast hexokinase at 30°, pH 7.5.

on high dilution did not occur when the dilution was carried out in the presence of other proteins (*e.g.* hemoglobin, serum albumin, insulin). The amount of protein required to prevent the loss of activity on dilution varied with the different proteins.

The data in Fig. 3 show that insulin (a purified amorphous preparation supplied by Eli Lilly and Co.) was the most effective of the proteins tested, complete protection being afforded by 10γ per 1.6 cc. of reaction mixture. Its effectiveness as a protective protein was not impaired by treatment with dilute alkali under conditions known to abolish its physiological activity (10). Reduction of the insulin with cysteine by the procedure of Wintersteiner (11), which also abolishes its physiological activity, caused a marked decrease in its

protective power. Human serum albumin (a 4-times crystallized sample kindly supplied by Dr. A. A. Green) was much less effective than insulin, more than  $100\gamma$  being required to protect the hexokinase completely. Solutions of crude yeast hexokinase (300 units per mg.), when inactivated by heating for 5 minutes at  $100^\circ$ , were about as effective as serum albumin in protecting the pure enzyme from loss of activity on dilution. Heat-inactivated crystalline hexokinase was by far the least effective as a protective protein,  $160\gamma$  being required for full protection. The effect of heating on the protective power has not been investigated quantitatively;  $100\gamma$  of heat-treated insulin or serum

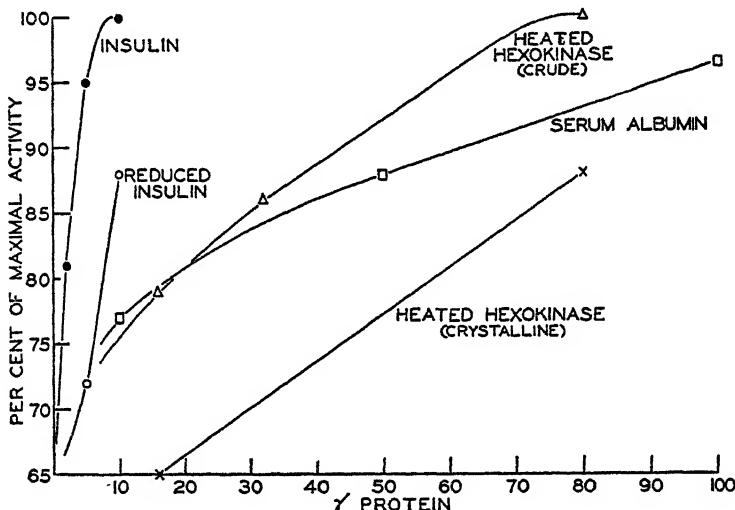


FIG. 3. Effects of various proteins on the activity of a dilute solution ( $6.7\gamma$  per 1.6 cc.) of crystalline hexokinase. The activity of the enzyme in the absence of protective protein was 65 per cent of that observed when the dilution was carried out in the presence of an excess of any one of the proteins tested. Temperature  $30^\circ$ , pH 7.5.

albumin showed the same protective action as the unheated controls. In addition to the proteins shown in Fig. 3, crystalline horse hemoglobin and the protein of a crude aqueous extract of rabbit muscle have been tested. The former showed maximal action in a concentration of  $100\gamma$  per 1.6 cc., but was not tested at lower concentrations.  $10\gamma$  of the muscle protein was about as effective as  $10\gamma$  of reduced insulin.

In Fig. 4, the effect of dilution on the activity of crystalline hexokinase is illustrated. In the absence of protective protein, the specific activity fell off markedly on dilution. When the dilution was carried out in the presence of an excess of insulin ( $100\gamma$ ), the specific activity was independent of the hexoki-

nase concentration. This amount of insulin was used as a routine in testing the activity of the pure enzyme at high dilutions.

It will be noted that the activity measurements in Fig. 4 were carried out at 15° instead of at the standard temperature of 30°. This was done in order to slow down the activity with the larger amounts of crystalline hexokinase to a conveniently measurable rate. The observed specific activity at 15° (1050 units per mg.) as compared to that at 30° corresponds to a temperature coefficient

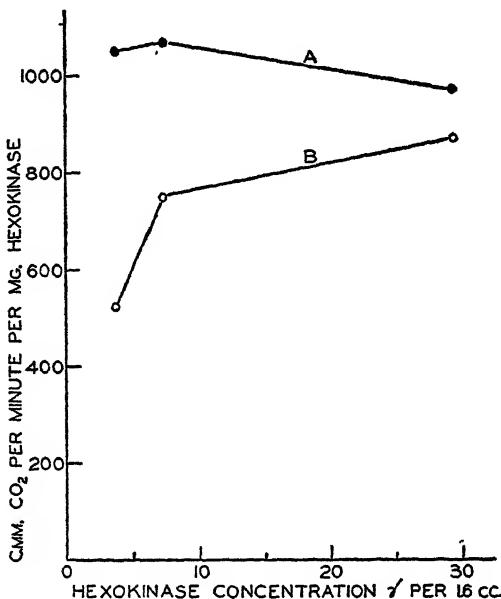


FIG. 4. Effect of dilution on the activity of crystalline hexokinase in the presence and absence of protective protein (insulin). Activity was determined at 15° at pH 7.3. Curve B shows the marked decrease in specific activity on dilution in the absence of protective protein. Curve A shows the protective action of 100 γ insulin per 1.6 cc. reaction mixture.

( $Q_{10^{\circ}}$ ) of 2.2. In another experiment in which the activity was measured at 0°, with the addition of sufficient NaHCO<sub>3</sub> (0.052 M) to maintain the pH at 7.5 at this temperature, a  $Q_{10^{\circ}}$  value of 1.9 was obtained for the range 0° to 30°. Even at 0°, the enzyme lost activity on high dilution, and this loss could be prevented by the presence of protective protein.

*Effect of Glucose on Inactivation by Proteolytic Enzymes.*—Solutions of the crystalline enzyme could be incubated in the absence of glucose without any loss of activity (Table IV), in contrast to crude preparations which lost activity rapidly unless glucose was added (Fig. 1). It seemed likely that the loss of activity in crude preparations was due to the action of proteolytic enzymes,

and if this was true, it was to be expected that glucose would protect hexokinase against proteolytic inactivation. This was tested in the experiments in Table IV. Solutions of crystalline hexokinase were almost completely (97 per cent) inactivated by incubation for 5 minutes at pH 7.6 and 20° with 100 $\gamma$  of crystalline trypsin,<sup>5</sup> when no glucose was present. When 0.17 M glucose was present, the inactivation after 5 minutes of incubation with trypsin amounted to only 17 per cent. Even after 20 minutes of incubation with this large amount of trypsin a protective action of glucose could be demonstrated. Hexokinase, when combined with glucose, appears therefore to be much more resistant to proteolytic inactivation than is the free enzyme.<sup>6</sup>

*Effect of Reducing Agents.*—Hexokinase does not appear to contain any highly reactive —SH groups which are essential to its catalytic activity. The

TABLE IV

*Inactivation of Crystalline Hexokinase by Trypsin and Its Prevention by Glucose*

4 samples were prepared, each containing 24 units of crystalline hexokinase in 0.1 cc. of 0.03 M potassium phosphate buffer pH 7.6, plus 0.2 cc. of the following additions: (1) 0.2 cc. H<sub>2</sub>O; (2) 0.1 cc. 0.5 M glucose + 0.1 cc. H<sub>2</sub>O; (3) 0.1 cc. 0.1 per cent crystalline trypsin + 0.1 cc. H<sub>2</sub>O; (4) 0.1 cc. 0.1 per cent crystalline trypsin + 0.1 cc. 0.5 M glucose. 0.1 cc. samples were removed after 5 and 20 minutes at 20° and tested immediately for hexokinase activity by the usual procedure.

Sample No.	Units of hexokinase activity found	
	After 5 min.	After 20 min.
1	7.2	7.8
2	7.7	8.4
3	0.2	0.0
4	6.0	3.0

crystalline enzyme is optimally active without the addition of cysteine or glutathione. In no case has it been possible to demonstrate a stimulatory or protective effect of these substances on hexokinase activity. They are ineffective in preventing the loss in activity of the crystalline enzyme which occurs on high dilution, and do not protect the crude enzyme against inactivation by heat or proteolytic action.

*Ultraviolet Absorption Spectrum.*—It has been reported recently that muscle

<sup>5</sup> Kindly supplied by Dr. J. Northrop.

<sup>6</sup> By taking advantage of this finding, it has been possible to effect a considerable purification of crude yeast hexokinase merely by allowing a concentrated solution to stand in the presence of glucose until the bulk of the protein has been digested away. The hexokinase and other remaining proteins can then be salted out with ammonium sulfate. By this simple procedure, the specific activity of a crude preparation has been increased from 100 to 400 units per mg. of protein.

hexokinase requires both guanine and dihydrocozymase for its activity and that the latter is present in a bound form (12). Yeast hexokinase does not require the addition of these substances for its activity and the ultraviolet absorption spectrum (Fig. 5) does not reveal their presence either at 255 or 340 m $\mu$ .

#### SUMMARY

1. A method is described for the isolation of hexokinase from baker's yeast. The method is based mainly on fractionation with alcohol and results

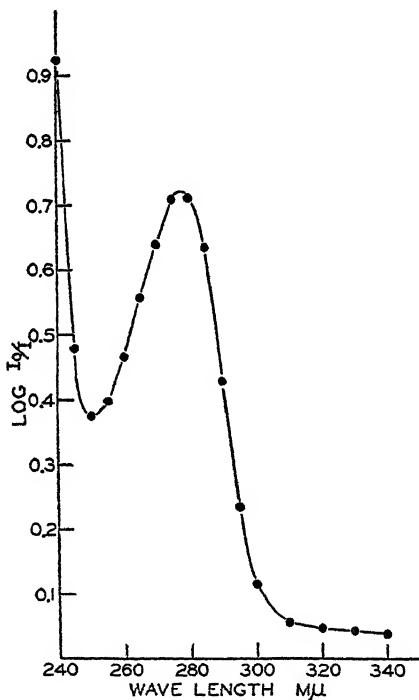


FIG. 5. Ultraviolet absorption spectrum of crystalline yeast hexokinase. Enzyme solution had been dialyzed against distilled water. Protein concentration 0.55 mg. per cc. Width of quartz cell 1 cm.

in a 30-fold increase in specific activity. The final product could be crystallized from ammonium sulfate without change in specific activity.

2. The enzyme catalyzes a transfer of phosphate from adenosinetriphosphate to glucose, fructose, or mannose, the relative rates with these three sugars being 1:1.4:0.3.

3. With glucose as substrate, the turnover number for the crystalline enzyme is 13,000 moles of substrate per  $10^5$  gm. of protein per minute at 30° and pH 7.5. The temperature coefficient ( $Q_{10^\circ}$ ) between 0 and 30° is 1.9.

4. Magnesium ions are necessary for the activity, the dissociation constant for the  $Mg^{++}$ -protein complex being  $2.6 \times 10^{-3}$ . Fluoride in concentrations as high as 0.125 M has no inhibitory effect on the enzyme when the  $Mg^{++}$  and orthophosphate concentrations are  $6.5 \times 10^{-3}$  M and  $1 \times 10^{-3}$  M, respectively.

5. The crystalline enzyme shows a loss in activity when highly diluted. This loss in activity can be prevented by diluting in the presence of small amounts of other proteins. Of the various protective proteins tested, insulin was the most effective, providing complete protection in a concentration of 6 micrograms per cc.; with serum albumin, a concentration of 60 micrograms per cc. was necessary. Thiol compounds (cysteine, glutathione) exerted no protective action.

6. The inactivation of the crystalline enzyme on incubation with trypsin can be prevented to a marked degree by the presence of glucose. The instability of crude preparations of yeast hexokinase may be attributed to the presence of proteolytic enzymes, since glucose or fructose has a remarkable protective effect on such preparations.

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# CRYSTALLINE HEXOKINASE (HETEROPHOSPHATESE)\*†§

## METHOD OF ISOLATION AND PROPERTIES

By M. KUNITZ AND MARGARET R. McDONALD

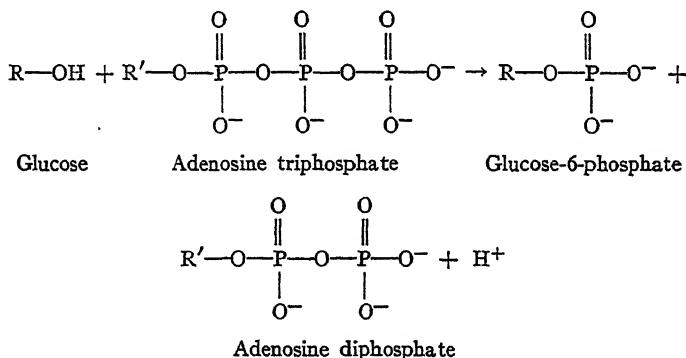
(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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### INTRODUCTION

Meyerhof (1) in 1927 obtained an "activator" from yeast which restored to aged muscle juice its lost ability to ferment glucose. In 1935 von Euler and Adler (2) found an enzyme in yeast which catalyzed the transfer of phosphorus from adenosine triphosphate to hexoses. They named the enzyme "heterophosphatase." Meyerhof (3) then established that his "activator" of 1927, which he renamed "hexokinase" had the same enzymatic property as von Euler's "heterophosphatase."

Colowick and Kalckar (4) in 1941 showed that hexokinase catalyzes the transfer of one phosphate group from adenosine triphosphate to glucose with the liberation of one hydrogen equivalent of acid. The reaction is represented as follows:



\* A preliminary note on the isolation of crystalline hexokinase and three other crystalline proteins from yeast has been published recently (Kunitz, M., and McDonald, M. R., *J. Gen. Physiol.*, 1946, 29, 143).

† This paper is based on work done for the Office of Scientific Research and Development under Contract No. OEMsr-129 with The Rockefeller Institute for Medical Research. The experiments referred to were first reported August 16, 1943.

§ The writers acknowledge with thanks the kind cooperation of Dr. Sidney P. Colowick.

This paper describes the isolation from baker's yeast of pure hexokinase in crystalline form. Crystalline hexokinase is a protein of the albumin type. It is crystallized at 5°C. in the presence of ammonium sulfate and dilute phosphate buffer pH 7.0. The crystals become relatively pure after two or three crystallizations as tested by solubility, electrophoresis, and ultracentrifuge measurements. The hexokinase activity of the crystals is associated with the protein nature of the material.

Solutions of crystalline hexokinase in dilute buffers of pH 4.5-7.5 are stable for several days when kept at a temperature of 5°C. or lower. At higher temperatures the hexokinase activity is rapidly lost. The loss in activity is accompanied by denaturation of the protein. The point of maximum stability is around pH 5.0 which is near the isoelectric point of the material. The elementary composition of crystalline hexokinase is that of a typical protein. It contains 0.11 per cent phosphorus which would indicate a minimum molecular weight of about 30,000. Sedimentation and diffusion measurements in acetate buffer pH 5.5 at 1°C. gave a molecular weight of 96,600.

#### *Method of Preparation of Crystalline Hexokinase from Baker's Yeast*

Meyerhof's (1) original method for preparing hexokinase from yeast consisted essentially in plasmolyzing baker's yeast by means of toluene and extracting the plasmolyzed yeast with water at 35°C. The active material was then precipitated in 50 per cent alcohol at 0°C. Berger, Stein, Colowick, and Cori (5) reported that purification of crude hexokinase by means of alcohol is more effective if carried out in a solution containing 1 per cent dextrose and 0.05 M acetate buffer pH 5.2-5.4.

Meyerhof's method of plasmolysis and of extraction with water was used as a starting step in the present work on the preparation of hexokinase. Advantage was also taken of Meyerhof's method of purification by means of alcohol as modified by Colowick and associates. The basis of the present method, however, is the technique of purification and crystallization of proteins by means of ammonium sulfate from concentrated protein solutions as developed by Northrop, Kunitz, and others for the isolation of crystalline enzymes.

The method consists essentially of the following steps:

1. Plasmolysis with toluene and extraction of the plasmolyzed yeast with water.
2. Concentration and fractionation by means of ammonium sulfate.
3. Removal of "inert" crystalline proteins.

Several crystalline proteins, including a crystalline yellow protein, appear during the process of fractionation with ammonium sulfate. These proteins do not possess hexokinase activity. The separation of these crystalline proteins, however, leads to a considerable improvement in the hexokinase activity of the remaining fractions.

4. Dialysis.
5. Purification by fractional precipitation with alcohol.

6. Crystallization in the presence of ammonium sulfate.
7. Recrystallization.

All operations, except when mentioned otherwise, are done at temperatures of 8–10°C. The pH of the preparations is tested by the drop method, by mixing 1 drop of solution with 1 drop of 0.01 per cent Clark indicator on a test plate and comparing the colors with those of drops of 0.1 M standard buffers mixed on the plate with the same indicators. The saturated ammonium sulfate is prepared at about 20°C.

The details of the method of preparation of crystalline hexokinase are as follows:—

1. *Plasmolysis and Extraction*.—25 pounds of fresh Fleischmann's baker's yeast is broken by hand into small fragments and then macerated by means of a wooden paddle in a large aluminum or enameled vessel with 6 liters of warm toluene of about 40°C. The vessel is placed in a water bath of about 45°C. The maceration is continued until the yeast is heated to 37°C. at which temperature the yeast rapidly liquefies and begins to "work." A rapid liberation of CO<sub>2</sub> takes place and the volume of the mixture increases considerably. It is left in the room for 2 to 3 hours and then cooled to 10°C. in an ice water bath. The thick mixture of plasmolyzed yeast and toluene is distributed in four 10 liter jars, 3 liters of distilled water of about 5°C. is added to each jar and mixed. The jars are left for 18 hours at 5°C. A layer of an emulsion of the toluene with yeast stromata gradually forms above the yeast-water suspension.

The yeast suspension is siphoned off from under the toluene-stromata emulsion and then filtered in the cold room at 8–10°C. with suction on four 32 cm. Büchner funnels with the aid of 100 gm. hyflo super-cel<sup>1</sup> per liter of fluid using Eaton-Dikeman No. 303 paper. The residue on each funnel is washed once with 1 liter of cold water. (The toluene is partly recovered by filtering the toluene-stromata emulsion with the aid of 100 gm. hyflo super-cel per liter.)

2. *Fractionation with Ammonium Sulfate*.—The clear filtrate and washings are brought to 0.5 saturation with solid ammonium sulfate (314 gm. per liter of filtrate) and the precipitate formed is filtered with suction with the aid of 10 gm. of standard super-cel, plus 10 gm. filter-cell per liter of solution. The residue is discarded. The clear filtrate is brought to 0.65 saturation by further addition of 99.3 gm. of solid ammonium sulfate to each liter of filtrate. The precipitate formed (called 0.6 fraction) is filtered with suction on No. 612 E. & D. paper on large funnels. The filtrate is brought to 0.7 saturation by the addition of 33.8 gm. of solid ammonium sulfate to each liter of filtrate. The precipitate formed (called 0.7 fraction) is filtered with suction after standing for 16 to 18 hours at a temperature not higher than 10°C. Both 0.6 and 0.7 fractions are used for isolation of hexokinase. The purification of the 0.7 fraction will be described first since it involves fewer steps and the yield of crystalline hexokinase obtainable is often greater than in the 0.6 fraction.

3. *Removal of "Inerl" Crystalline Proteins*.—(a) Isolation of crystals of "yeast

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<sup>1</sup> Supplied by Johns-Manville Corporation, New York.

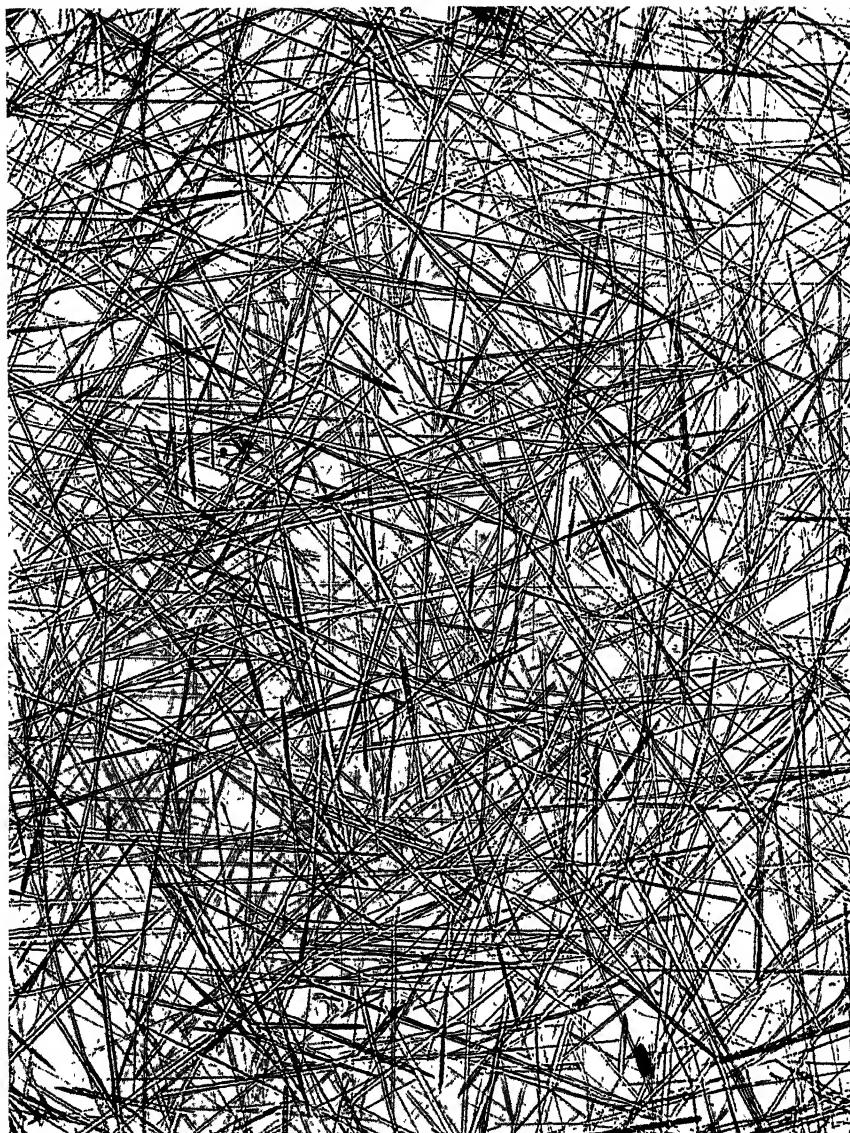


FIG. 1. Crystals of yeast protein No. 2.  $\times 129.5$ .

protein, No. 2." Each gram<sup>2</sup> of the 0.7 fraction is dissolved in 2 ml. cold water, the pH adjusted to 7.4 with 1 M sodium hydroxide, then enough saturated ammonium

<sup>2</sup> This expression is used to denote the relative volume of solvent in which the precipitate is dissolved. It does not mean that each gram is dissolved separately.

sulfate added with stirring to trace of turbidity. It generally requires about 2.5 ml. ammonium sulfate per gm. filter cake. The solution is kept in an ice bath during this operation and then stored at about 5°C. for 6 to 8 days. A gel of very fine crystals of yeast protein, No. 2, is gradually formed. This is filtered off with the aid of 5 gm. standard super-cel per 100 ml. of solution.<sup>3</sup> The filtrate is brought to 0.85 saturation with solid ammonium sulfate, added slowly with stirring (21.5 gm. per 100 ml. of filtrate). The precipitate formed is filtered with suction. Filter cake = fraction 0.71.

(b) Isolation of crystals of "yeast protein, No. 3." Each gram of filter cake of fraction 0.71 is dissolved in 0.5 ml. cold water at 2 – 5°C. Saturated ammonium sulfate is added to trace of turbidity, then 1 M sodium hydroxide to pH 7.5. The solution is left at 5°C. for 5 to 6 days. Prismatic crystals of yeast protein, No. 3 (Fig. 2), are gradually formed. The solution is centrifuged at 5–10°C.<sup>4</sup> The supernatant solution is diluted with 2 volumes of cold 0.65 saturated ammonium sulfate pH 7.2 (containing 2 ml. 5 M sodium hydroxide per liter), 10 gm. of standard super-cel and 43 ml. saturated ammonium sulfate are then added for each 100 ml. of original supernatant solution. The ammonium sulfate solution is added slowly with stirring. The precipitate formed is filtered with suction, resuspended twice in a volume of 0.65 saturated ammonium sulfate of pH 7.2, equal to that of the first filtrate, and refiltered each time with suction. Combined filtrates are brought with solid ammonium sulfate to 0.85 saturation (143 gm. per liter) and filtered with suction. Filter cake = fraction 0.72.

(c) Isolation of crystals of "yeast yellow protein." Each gram of 0.6 fraction is dissolved in 1 ml. of cold water and 1 ml. saturated ammonium sulfate is added; a slight precipitate generally forms. The turbid solution is stored for 20 to 24 hours at 5°C. It is then centrifuged and the residue is discarded. The filtrate is titrated with 0.5 N NaOH to pH 7.2 and stored at 5°C. for 5 to 7 days. The gelatinous precipitate formed is removed by filtration with the aid of 10 gm. hyflo super-cel per 100 ml. of solution. (The residue may yield No. 2 crystals when treated as described in footnote 3.) The clear filtrate is brought to 0.85 saturation by the slow addition of 233 ml. saturated ammonium sulfate per 100 ml. filtrate, and filtered with suction. Yield 200 to 300 gm. filter cake. This is dissolved in one half volume of H<sub>2</sub>O, saturated ammonium sulfate is added to trace of turbidity. The solution is then titrated with 1

<sup>3</sup> Recrystallization of yeast protein, No. 2.: The protein is extracted from the super-cel with cold water of about 5°C. and is reprecipitated with solid ammonium sulfate at 0.85 saturation. The precipitate is dissolved in about 3 volumes of cold water, 8.0 ml. saturated ammonium sulfate is added for every 10 ml. of water, and the solution is titrated with 0.5 N NaOH to pH 7.4 and stored for 7 days or longer at about 5°C. Gradual crystallization of long needles takes place (Fig. 1).

Yeast protein, No. 2, can also be recrystallized at pH 4.3 in 0.5 saturated ammonium sulfate at 20–25°C. Under these conditions the crystals appear in the form of hexagonal and rhomboid plates.

<sup>4</sup> Recrystallization of yeast protein, No. 3.: The centrifuged residue of crystals is dissolved in about 2 volumes of cold water and 2.5 volumes of saturated ammonium sulfate is added; the clear solution is titrated with 0.5 N NaOH to pH 7.4 and stored for several days at 5°C. Gradual crystallization of well formed prisms takes place.

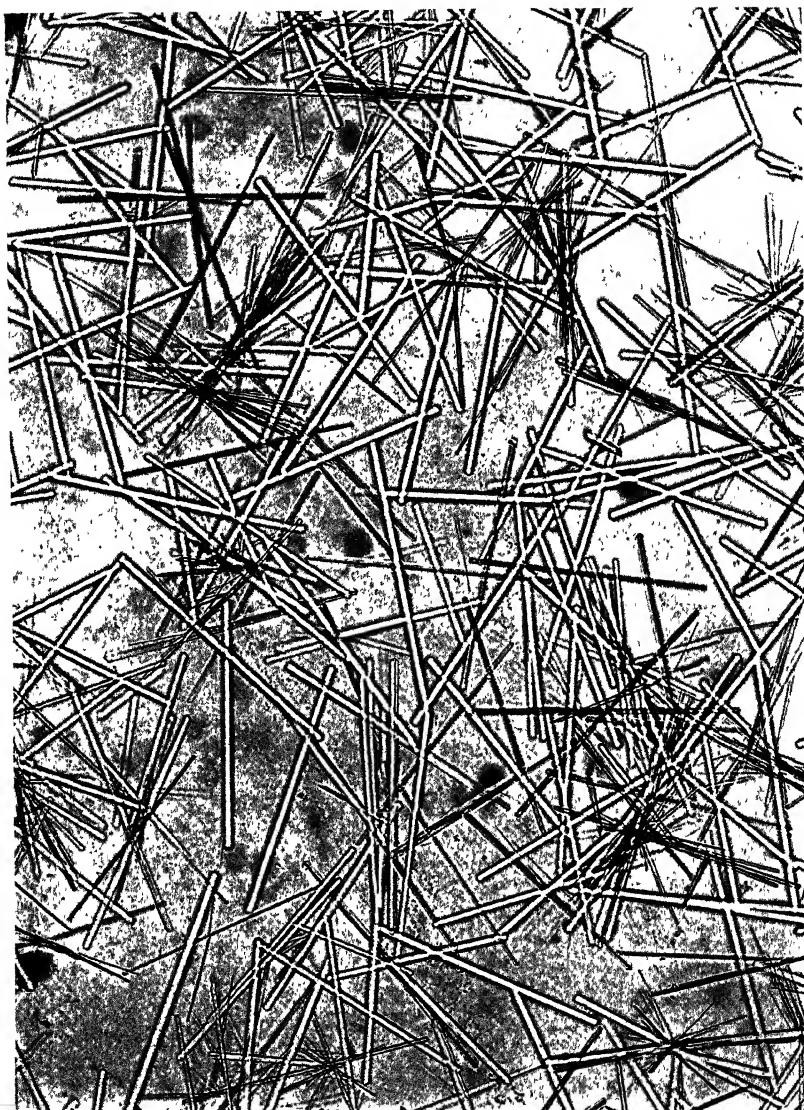


FIG. 2. Crystals of yeast protein, No. 3.  $\times 129.5$ .

n NaOH to pH 7.5, and stored at 6°C. Crystals of a yellow protein (Fig. 3) gradually form. These are centrifuged after 1 to 2 weeks.<sup>5</sup>

<sup>5</sup> Recrystallization of the yeast yellow protein.: The centrifuged residue of crystals is dissolved in an equal volume of cold water and one half volume of saturated ammo-

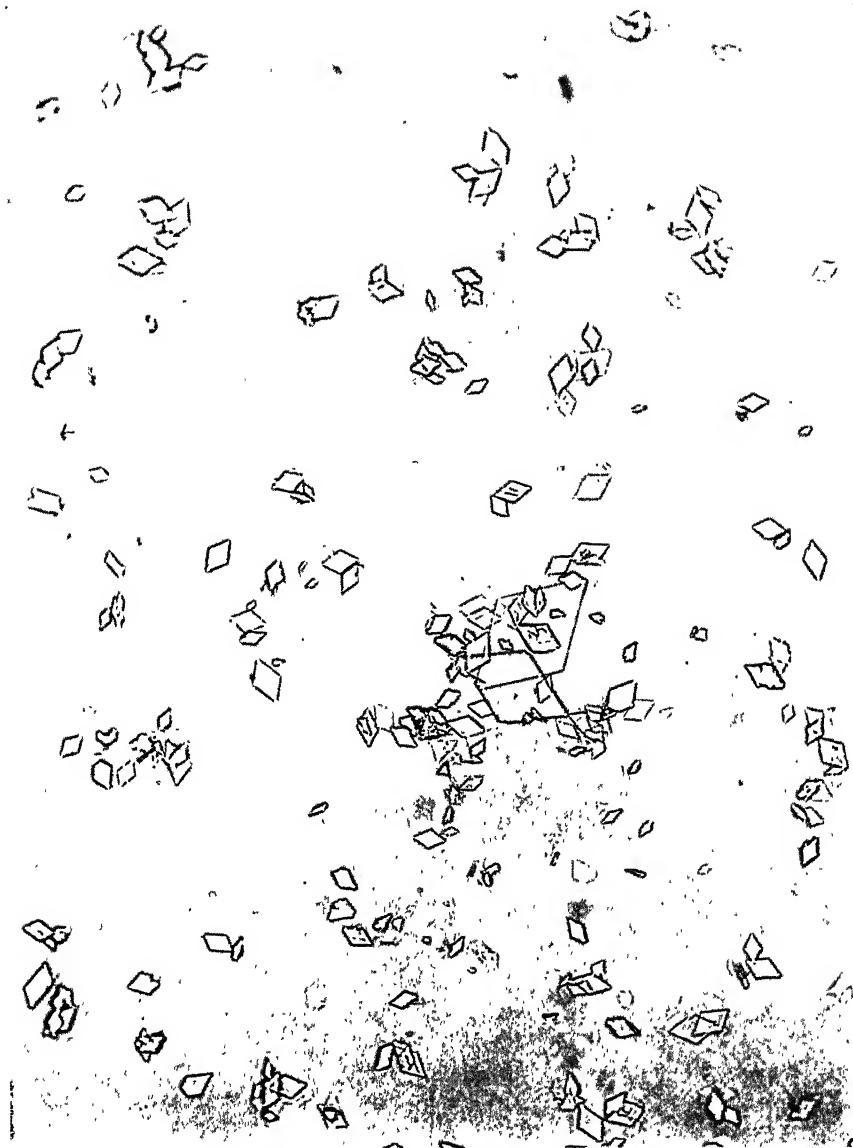


FIG. 3. Crystals of yeast yellow protein.  $\times 128$ .

The supernatant solution is diluted with 2 volumes of cold 0.65 saturated ammonium sulfate of pH 7.2. 10 gm. of standard super-cel and 43 ml. saturated ammonium sulfate is gradually added. The solution is titrated with 0.5 N NaOH to pH 7.5 and stored at 5°C. for several days. Rhombohedral crystals of the yellow protein gradually form.

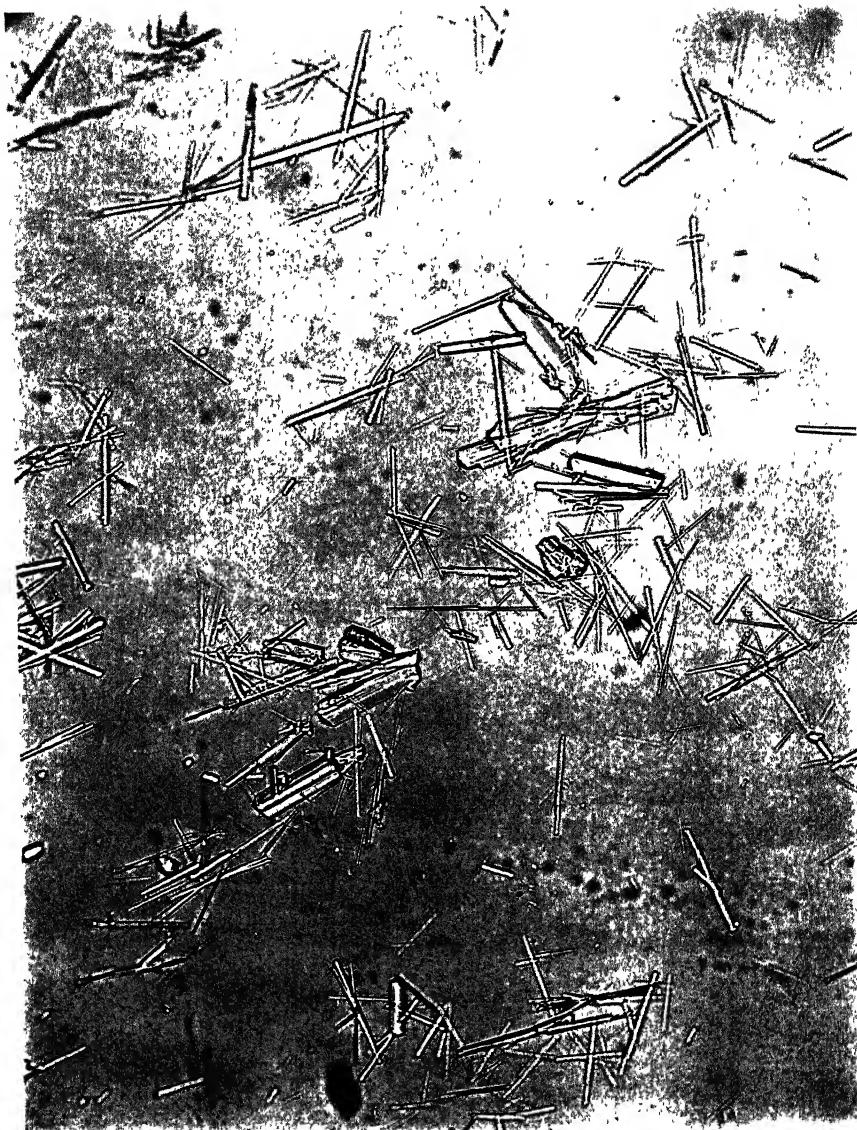


FIG. 4a. Crystals of hexokinase.  $\times 128.2$ .

sulfate are added for each 100 ml. of the original supernatant solution. The suspension is filtered with suction on No. 3 paper. The precipitate is resuspended twice in a volume of 0.65 saturated ammonium sulfate of pH 7.2, equal to that of the first filtrate, and refiltered each time with suction. Combined filtrates are brought with solid ammonium sulfate to 0.85 saturation (143 gm. per liter) and filtered with suction.

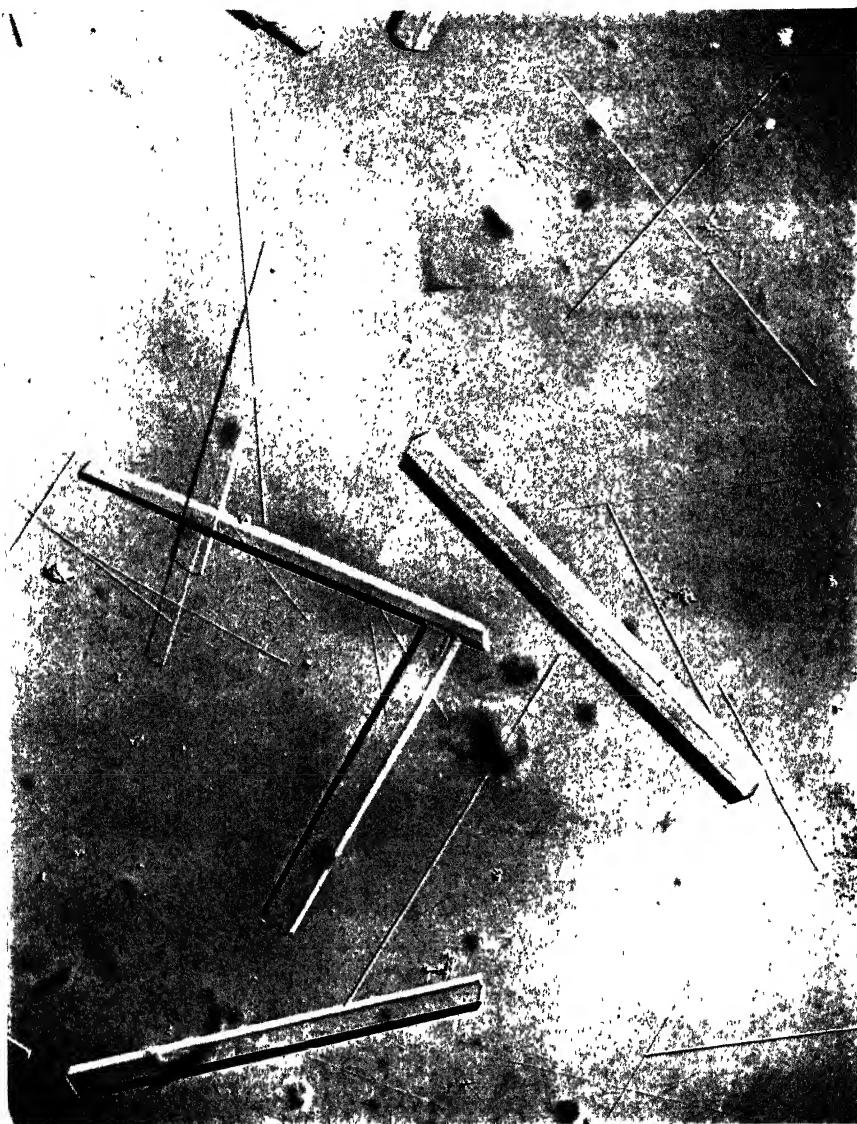


FIG. 4b. Large crystals of hexokinase.  $\times 116$ .

Filter cake about 50 gm.—fraction 0.62—is treated exactly as described in section 3b to yield fraction 0.63. A certain amount of No. 3 crystals are generally formed during this step.<sup>6</sup>

<sup>6</sup> If the activity of fraction 0.72 is less than 50 hexokinase units per mg. protein, then step 3b should be repeated. The final precipitate, fraction 0.72, 0.73 or 0.63, as

4. *Dialysis in Dextrose Solution.*—Each gram of filter cake of fraction 0.72, 0.73, or 0.63, as the case may be, is dissolved in 1 ml. of 1 per cent dextrose. The solution is transferred into a collodion bag provided with a large glass bead and is dialyzed in a rocking machine for 18 hours at about 5°C. against slowly running 1 per cent dextrose.

5. *Fractional Precipitation with Alcohol.*—(a) The dialyzed solution is diluted with cold 1 per cent dextrose to a volume equal in milliliters to 4.75 times the original weight in grams of the filter cake in step 4, and 1 ml. 1 M acetate buffer pH 5.4 is added per 19 ml. of solution. The solution is cooled in a freezing mixture bath to about -2°C. and 35.7 ml. cold 95 per cent alcohol added gradually with stirring to each 100 ml. of solution to a concentration of 25 per cent. The precipitate formed is centrifuged off at about 5°C. The supernatant fluid is measured, cooled again to -2°C., and more 95 per cent alcohol added to a final concentration of 50 per cent (55.5 ml. per 100 ml. supernatant). The suspension is centrifuged, and the supernatant discarded. The residue is resuspended in a volume of cold 1 per cent dextrose equal to 2 times the weight of the filter cake in step 4 and recentrifuged. The clear supernatant is brought to pH 5.4 by the addition of 5 ml. 1 M acetate buffer of that pH to 100 ml. of solution and the alcohol fractionation is repeated. (This repetition is unnecessary if the activity of the material before dialysis is above 100 units per mg. protein. In that case the residue left after centrifugation of the 50 per cent alcohol is suspended not in dextrose but in a volume of cold water equal to 5 times the weight of the filter cake in step 4. The suspension is then centrifuged and the supernatant solution is treated with ammonium sulfate as described in step 5b.)

(b) The final residue left after centrifugation of the 50 per cent alcohol mixture is resuspended in a volume of cold water equal to twice the weight of filter cake before dialysis in step 4. The suspension is recentrifuged. The clear supernatant fluid is brought with solid ammonium sulfate to 0.90 saturation (66 gm. per 100 ml.). The precipitate formed by the addition of ammonium sulfate is filtered with suction. The filtration generally takes 1 or 2 days.

6. *Crystallization.*—Each gram of filter cake obtained from step 5b is dissolved in 1 ml. 0.1 M phosphate buffer pH 7.0 at about 3°C. and 1 ml. saturated ammonium sulfate is added slowly. If a heavy precipitate is formed then a few drops of phosphate buffer are added to incipient clearing. The solution is centrifuged. The clear supernatant solution is left at about 5°C. Crystals in the form of long prisms or fine needles (Figs. 4a and 4b) gradually appear. Seeding hastens the crystallization, as usual.

7. *Recrystallization.*—The suspension of crystals is centrifuged after 7 to 10 days. The residue is dissolved in a minimum amount of cold 0.1 M phosphate buffer pH 7.0 and a volume of saturated ammonium sulfate is added equal to 1.4 volumes of the buffer used. The solution is left at 5°C. for crystallization which is usually completed in 2 to 3 days. The crystals are centrifuged or filtered with suction.

The yield of crystalline hexokinase varies considerably with the individual lots of yeast delivered to the laboratory, perhaps because of differences of age of

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the case may be, is used for dialysis and alcohol fractionation as described in steps 4 and 5.

the yeast. Some lots even fail to yield any hexokinase crystals. It is advantageous to carry through the purification of lots of 25 pounds of yeast to step 4 and to store the filter cakes (fractions 0.63, 0.72, and 0.73) at 5°C. until an accumulated stock of about 100 gm. is obtained for further treatment. In case hexokinase crystals fail to appear in the crystallization mixture within 7 to

TABLE I

Preparation	Filter cake	Total hexokinase activity units	Specific activity. Hexokinase units per mg. protein	Yield
Original aqueous extract + washing from 25 lb. yeast	gm.	4-8 million	About 20	6,000,000 = 100
Fraction 0.6	400-600	2-3 million	25-30	40
Fraction 0.7	150-250	1-2 million	25-30	25
Fraction 0.63	5-20	200,000-300,000	100-170	4
Fraction 0.72	30-70	400,000-500,000	50-120	8
Fraction 0.63	After alcohol fractionation	100,000	250	1.7
Fraction 0.72	" "	120,000	300	2.0
First crystals (combined 0.63 and 0.72)	Mg. protein			
First mother liquor	167	150,000	900	2.5
Second crystals		33,000	100	
Second mother liquor	80	90,000	1,100	1.5
Third crystals		22,000	300	
Third mother liquor	38	54,000	1,400	0.9
Fourth crystals		27,000	950	
Fourth mother liquor			1,350	
Fifth crystals			1,200	
Fifth mother liquor			1,440	
			1,320	

10 days the solution is then brought to 0.85 saturation with saturated ammonium sulfate and filtered with suction. The filter cake is reworked through steps 3b to 7.

The total yield of hexokinase can be increased by reworking the various inert protein crystals and super-cel residues of step 3, each according to its place in the general scheme.

The extent of purification of an average lot of 25 pounds of yeast is given in Table I. The specific activity of the various protein fractions is gradually raised on purification. The greatest rise, however, takes place on crystalliza-

tion. The specific activity of the first crystals is more than double that of the protein solution from which they were crystallized, while the protein left in the first mother liquor has a specific activity of only 11 per cent of that of the crystals. On further recrystallization the specific activity of the crystalline protein is gradually raised. It reaches a constant value after 2 or 3 recrystallizations. The specific activity of the protein in the mother liquor is lower than that of the crystals, the difference, however, becoming less and less as crystallization is repeated.

#### *Tests of Purity of Crystalline Hexokinase*

**1. Repeated Crystallization.**—The new protein has been recrystallized 5 times. The hexokinase activity per milligram of protein reached a constant value after

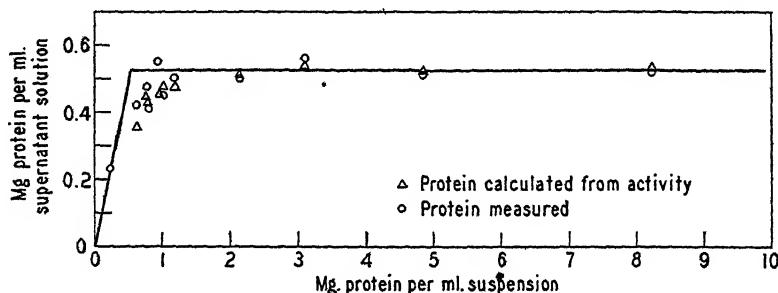


FIG. 5. Solubility curve.

two recrystallizations, while the activity of the mother liquors approached the same constant value after the third crystallization (see Table I).

**2. Solubility Curve.**—The purity of a crystalline protein is conveniently tested by measuring the solubility of the crystals in a suitable solvent in the presence of increasing amounts of crystals in suspension. A curve is plotted of the amount of protein dissolved *vs.* the total amount of protein in suspension. The plotted curve is compared with the theoretical phase rule curve for a pure substance (10).

Fig. 5 shows the result of a solubility test on the crystals of the hexokinase protein which has been recrystallized five times.

The crystals were first washed several times by centrifugation at 5°C. with a solution of ammonium sulfate of about the same composition as the solution used for crystallization, namely 2.5 M ammonium sulfate in 0.05 M phosphate buffer pH 7.0. Increasing amounts of the washed crystals were pipetted into 2 ml. test tubes. A glass bead was placed in each tube. The tubes were filled with 2.5 M ammonium sulfate in 0.05 M phosphate buffer pH 7.0 and stoppered. The test tubes were then attached by means of rubber bands on the face of a 5 inch wheel mounted on the shaft of a reducing gear which was slowly driven by a small motor. The rotation of the wheel at the

speed of 5 to 10 R.P.M. brought about continuous rolling of the glass bead along the side of each tube and thus kept the suspension stirred continuously. The suspensions were rotated for 18 hours at 5°C. Samples of each suspension were analyzed for protein, the rest of the material was centrifuged at 5°C., and the clear supernatant fluids were analyzed both for protein and hexokinase activity.

The solid lines in Fig. 5 represent the theoretical solubility curve of a pure substance. The experimental points fall close to the theoretical lines except near the point of their intersection. The irregularity in that region may be due to the presence of a small amount of impurities or denatured protein formed during the stirring as evidenced by the presence of fine strings and broken films of protein.

**3. Electrophoresis.**—The mobility of five times recrystallized hexokinase protein in the Tiselius apparatus was measured by Dr. A. Rothen. Measurements were made at pH 5.6 and 6.8. In both cases the protein was negatively charged and moved as a single electrophoretic component as shown by the uniformity and sharpness of the moving boundary.

**4. Sedimentation Studies by Means of the Ultracentrifuge.**—Tests by Dr. A. Rothen show that when centrifuged at pH 5.6 the protein is homogeneous to a high degree. At pH 6.0, however, a double boundary appears. This abnormality may be due to an effect of ultracentrifugation on the homogeneity of the hexokinase protein.

#### *The Protein Nature of Crystalline Hexokinase*

That the hexokinase activity of the crystals is associated with the protein nature of the material follows directly from the fact that the hexokinase activity per milligram of *protein* remains constant on repeated crystallization. Also, the solubility experiment showed that the dissolved material consisted of protein of the same specific activity as the bulk of the material. Additional evidence on the protein nature of the hexokinase is shown by studies on the stability of crystalline hexokinase. Inactivation is accompanied by denaturation of the protein. Hexokinase is inactivated in the presence of a small amount of trypsin. The inactivation is a gradual process and is accompanied by a loss of protein as tested by precipitation in 2.5 per cent trichloracetic acid. The inactivation, however, proceeds at a rate faster than the rate of digestion of the protein. Crystalline chymotrypsin does not appear to affect crystalline hexokinase.

#### *Stability of Crystalline Hexokinase*

**1. Effect of Temperature and pH.**—Crystalline hexokinase dissolved in dilute buffers of pH 4.5–7.5 is stable for 2 to 3 days when kept at a temperature of 5°C. or lower. At temperatures above 5°C. the enzymatic activity is gradually lost. The rate of inactivation increases rapidly with increase in temperature.

The rate of inactivation varies also with the pH of the solution. Hexokinase is most stable at about pH 5.0 which is near its isoelectric point. The inactivation in the region of pH 4.5-7.5 is not accompanied by any significant hydrolysis of the protein. The protein is denatured, however, and precipitates out when the solution containing the inactivated material is brought to pH 5.0.

*2. Effect of Various Substances on the Stability of Hexokinase at pH 7.0 and 27°C.*—The stability of a 0.0025 per cent solution of hexokinase in 0.02 M phosphate buffer pH 7.0 when stored at 26-27°C. is increased in the presence of certain sugars and also in the presence of glycine. The order of effectiveness of the various sugars tested is shown in Table II.

TABLE II

Concentration of sugar in hexokinase solution, per cent.....	1.0	0.02	0.001	0
	Per cent activity left after 20 hrs. at 27°C.			
Dextrose.....	74	70	6	0-5
Mannose.....	68	65		
Sucrose.....	29	13		
Fructose.....	17	0		
Galactose.....	6	0		
Maltose.....	0	0		
Glycine.....	57	22 in 0.1 per cent glycine		

The order of the effectiveness of sugars as stabilizers of hexokinase does not correspond to the order of their effectiveness as acceptors of phosphorus from adenosine triphosphate. The order in that case is:

Dextrose.....	100 per cent
Fructose .....	100 " "
Mannose .....	54 " "
Galactose .....	0 " "
Maltose.....	0 " "
Sucrose .....	Trace...

#### *• Physicochemical Properties of Crystalline Hexokinase*

*1. Elementary Analysis.*—An aqueous solution of five times recrystallized hexokinase protein was dialyzed in a collodion bag with stirring against slowly running distilled water for 24 hours at about 5°C. The dialyzed protein solution was then frozen in dry ice-methyl cellosolve mixture and evaporated to dryness under vacuum while frozen.

The chemical analysis was carried out by Dr. A. Elek of The Rockefeller Institute. The results of analysis are as follows:—

Carbon .....	52.16 per cent
Hydrogen .....	7.08 " "
Nitrogen .....	15.62 " "
Phosphorus .....	0.11 " "
Sulfur .....	0.91 " "
Ash .....	0.36 " "

2. Isoelectric Point of Crystalline Hexokinase by Cataphoresis.—Measurements were made of the rate of cataphoretic migration (6) of collodion and of quartz particles which had been soaked for a few minutes in 0.5 per cent hexoki-

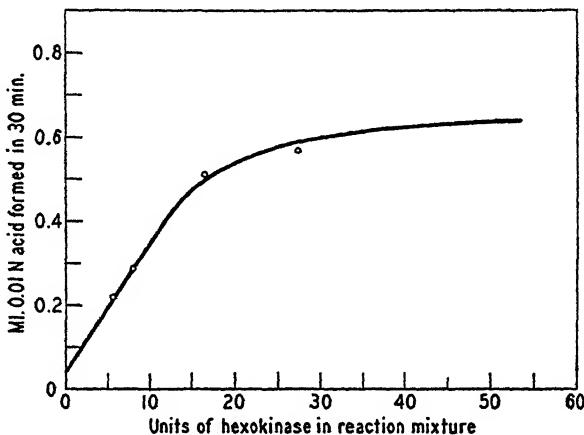


FIG. 6. Hexokinase activity curve.

nase solution and then suspended in 0.02 M buffer solutions. The region of minimum mobility was found to be at pH 4.5-4.8.

3. Ultracentrifuge Data by Dr. Rothen.—Diffusion constant ( $D_{20}^0$ ) at 1°C. measured in acetate buffer pH 5.5 =  $2.9 \times 10^{-7}$  cm.<sup>2</sup> sec.<sup>-1</sup>.

Sedimentation constant ( $S_{20}^0$ ) at same pH and temperature =  $3.1 \times 10^{-13}$  cm. sec.<sup>-1</sup> dyne<sup>-1</sup> gm.

Molecular weight at pH 5.5 calculated from the diffusion and sedimentation constants = 96,600.

The specific volume of the material was assumed to be 0.740 at 1°C. which is the usual value for a protein.

#### *The Enzymatic Properties of Crystalline Hexokinase*

1. Hexokinase Activity Measurement.—The hexokinase activity is measured by the rate of formation of free acid (4) during the process of the catalytic transfer of phosphorus from adenosine triphosphate to dextrose under standardized conditions. The amount of acid formed is determined by direct titration. Fig. 6 shows that the rate of formation of acid is nearly proportional to

the concentration of hexokinase over a range extending to the first 75 per cent of the ultimate amount of acid formed at the end of the reaction.

*2. The Hexokinase Unit.*—1 unit of hexokinase is defined as the amount of enzyme which catalyzes the formation of  $1 \times 10^{-8}$  hydrogen equivalents of acid per minute at 5°C. and pH 7.5, in a standard reaction mixture. This is equivalent to the liberation of about 1 mm<sup>3</sup>. of CO<sub>2</sub> from NaHCO<sub>3</sub> per minute at 25°C. when the acid formed is determined by the usual manometric method.

### 3. Technique of Acitivity Measurement.—

*Solutions.*—Stock solution of ATP (kindly suggested by Dr. Sidney P. Colowick).

0.35 gm. of dibarium adenosine triphosphate, C<sub>10</sub>H<sub>12</sub>O<sub>13</sub>N<sub>5</sub>P<sub>3</sub>Ba<sub>2</sub>, prepared from fresh calf muscle by the method of Kerr (7), is dissolved in 5 ml. 0.2 M hydrochloric acid, then 0.25 gm. of sodium sulfate (anhydrous) is added. The precipitate of barium sulfate is centrifuged off and washed once by centrifugation with 2 ml. of water. The combined supernatant solutions are neutralized to pH 7.5 with 1.0 M sodium hydroxide and the volume is made up to 10 ml. The solution is stable for several weeks when stored at 5°C.

Stock solution of 5 per cent dextrose (Pfanstiehl) stored with a crystal of thymol at 5°C.

Stock of 0.1 per cent phenol red in water containing 5.7 ml. 0.05 M sodium hydroxide per 100 ml.

*Solution A.*—20 ml. of stock solution of ATP + 3 ml. of 0.05 M magnesium chloride. Stored with a crystal of thymol at 5°C.

*Solution B.*—8 ml. 5 per cent dextrose + 3 ml. 0.5 M Sörensen's phosphate buffer pH 7.5 + water to 25 ml. Stored with thymol at 5°C.

*Solution C.*—2.5 ml. of stock of 0.1 per cent phenol red made up with water to 100 ml.

*Standard 0.01 M Sodium Hydroxide Solution.*—It contains 6.25 ml. of stock of 0.1 per cent phenol red per liter of solution.

*Standard 0.01 M Hydrochloric Acid.*—

*Reaction Mixture Used for Activity Measurements.*—

0.5 ml. solution A +

0.5 ml. solution B +

0.5 ml. solution C.

Mixed in test tubes 1.5 x 12 cm.

*Measurement.*—The test tube containing the reaction mixture is cooled to 5°C., then 0.5 ml. sample of hexokinase dissolved in ice-cold water is added. The mixture is adjusted immediately with 0.01 M sodium hydroxide or hydrochloric acid from micro-burettes to the color of a standard of pH 7.5, and then titrated with 0.01 M sodium hydroxide to the color of the standard after standing for 30 minutes at 5°C. The color standard consists of 2.5 ml. 0.1 M phosphate buffer pH 7.5 + 0.5 ml. of solution C (phenol red). The hexokinase units in the 0.5 ml. sample added are then read off the curve (Fig. 6).

### 4. Studies of the Enzymatic Action of Crystalline Hexokinase.—

(a) The effect of temperature. Fig. 7 shows the curves for the rate of the

reaction between adenosine triphosphate and dextrose at 5, 15, and 25°C. in the presence of a constant amount of crystalline hexokinase (2.5 hexokinase units). The rate of reaction was measured by the rate of formation of free acid in the reaction mixture. The temperature coefficient was estimated from the initial slopes of the curves and is about 2 per 10°C.

(b) Effect of crystalline hexokinase protein on the reaction between adenosine triphosphate and various sugars. The dextrose in the reaction mixture was replaced by the following sugars:

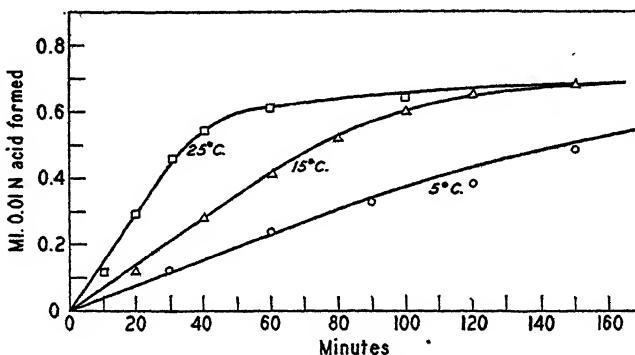


FIG. 7. Effect of temperature.

TABLE III

Molar concentration of magnesium chloride in solution A.	0.04	0.02	0.01	0.005	0.0025	0.00125	0
0.01 N acid formed in 30 min. at 5°C., ml.....	0.50	0.49	0.47	0.31	0.25	0.16	0.06

Pentoses: *l*-arabinose, *d*-xylose, *L*-rhamnose

Hexoses: *d*-dextrose, *d*-fructose, *d*-mannose, *d*-galactose

Disaccharides: sucrose, *d*-lactose, maltose, trehalose.

Trisaccharides: raffinose.

It was found that only dextrose, fructose, and mannose react with adenosine triphosphate. The rate of reaction in the presence of 15 hexokinase units is the same for dextrose and fructose and only about half as much for mannose. The samples of sugars used were mostly Pfanzstiehl, c. p.

(c) Effect of magnesium ions on hexokinase activity. Crystalline hexokinase requires for its catalytic action the presence of magnesium ions. This is shown in Table III. The usual reaction mixture was used except for varying the concentration of magnesium chloride in solution A.

(d) Effect of inorganic phosphate ions. Crystalline hexokinase does not require the presence of inorganic phosphate ions as shown in Table IV. The

usual reaction mixture was used except for varying the concentration of phosphate buffer in solution B.

(e) Effect of varying the concentration of the substrates. Within certain limits of concentrations of adenosine triphosphate and of dextrose the rate of reaction is practically independent of their concentrations, as shown in Tables V and VI.

TABLE IV

Molar concentration of phosphate buffer in solution B.....	0.20	0.10	0.05	0.025	0.0125	0
0.01 N acid formed in 30 min. at 5°C., ml..	0.38	0.42	0.47	0.44	0.43	0.40

TABLE V  
*Effect of Varying the Concentration of ATP*

ML solution A in 2.5 ml. final reaction mixture. Concentration of mag- nesium chloride 0.004 M.....	1.0	0.75	0.50	0.35	0.20
0.01 N acid formed in 30 min. at 5°C., ml.....	0.34	0.38	0.39	0.38	0.26

TABLE VI  
*Effect of Varying the Concentration of Dextrose*

Dextrose in 2 ml. final reaction mixture, per cent.....	0.8	0.6	0.4	0.2
0.01 N acid formed in 30 min. at 5°C., ml.....	0.51	0.51	0.50	0.50

*The Extent of Reaction*

The extent of the reaction, as determined by the final amount of acid formed, varied slightly with the samples of adenosine triphosphate used, depending on their percentage content of pure ATP. The rate of the reaction, however, was found not to vary with the sample used, in agreement with the results given in Table V. The final amount of acid formed is equivalent approximately to one-third of the phosphorus content of the adenosine triphosphate in the reaction mixture, thus confirming the findings of Colowick and Kalckar (4). Crude hexokinase preparations gave rise to the formation of acid equivalent to about two-thirds of the phosphorus content of the ATP.

*Protein Determination*

*Protein by Turbidity.*—5 ml. sample of protein is mixed with 5 ml. 5 per cent trichloroacetic acid. The mixture is left at about 20°C. for 1 hour. The tur-

bidity formed is compared in a photoelectric colorimeter with that of a suspension of the same protein material of a known concentration, as determined by Kjeldahl analysis. A more convenient way is to draw a calibration curve for the turbidity of several concentrations of the standard protein solution as read against a suitable glass disk. This avoids the necessity of preparing fresh standards. This method covers the range of 0.02 to 0.8 mg. protein per ml.

*Colorimetric Method* (according to Herriott (8)).—1 ml. sample containing 0.2 to 1.2 mg. protein + 1 ml. 0.0025 M cupric sulfate + 8 ml. 0.5 M sodium hydroxide + 3 ml. of 1:3 dilution of Folin's phenol reagent (9) in water. The color developed is measured after 3 to 7 minutes and compared with that of a standard as described for the turbidity method.

#### SUMMARY

1. Crystalline hexokinase has been isolated from baker's yeast.
2. Crystalline hexokinase is a protein of albumin type of a molecular weight of 96,000. Its isoelectric point is at about pH 4.8.
3. The method of isolation consists in separating the proteins of an aqueous extract of toluene-treated yeast by means of fractional precipitation with ammonium sulfate and with alcohol.
4. The procedure involves also the separation of several crystalline proteins, including one yellow crystalline protein, which do not possess hexokinase activity. The biological and the physicochemical properties of these proteins are still under investigation.
5. The crystallization of hexokinase proceeds at about 5°C. in the presence of ammonium sulfate and dilute phosphate buffer pH 7.0.
6. Crystalline hexokinase becomes relatively pure after 2 or 3 recrystallizations as tested by solubility, sedimentation in the ultracentrifuge, and electrophoresis. The enzymatic activity remains constant on repeated crystallization.
7. The enzymatic activity is associated with the protein nature of the material. Inactivation is accompanied by denaturation of the protein.
8. Crystalline hexokinase is relatively stable when stored in the form of crystalline filter cake. Solutions of hexokinase in dilute buffers are most stable at pH 5.0.
9. Crystalline hexokinase requires the presence of magnesium ions for its catalytic activity.

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# SOME PROPERTIES OF THE YEAST YELLOW PROTEIN

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The yellow protein crystallized by Kunitz and McDonald (7) from yeast has been examined for some of its properties. The crystalline preparations employed were kindly placed at my disposal by these authors. The yellow color of the preparation appears to be largely due to a flavin prosthetic group, though another yellowish prosthetic group also appears to be present. No enzymatic function for this protein preparation has as yet been found.

## EXPERIMENTAL

The preparations of the yellow protein were received as a paste containing ammonium sulfate. In the first preparation received, attempts to dialyze away this salt resulted in a loss of color and denaturation of the protein. All subsequent experiments were, therefore, performed on solutions of the protein made by dissolving the paste in water. A clear lemon-yellow-colored solution was thus obtained.

The absorption spectrum of such a solution, as measured in a Beckman spectrophotometer, is shown in Fig. 1 by the curve labeled "Oxidized." A peak may be observed in the region of  $\lambda$  450 m $\mu$ . If, to this solution, a little solid dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) is added, the yellow color fades pronouncedly but does not entirely disappear. This solution was overlaid with mineral oil in order to prevent its reoxidation by air and its absorption spectrum was measured. The curve obtained is the one shown in Fig. 1 labeled "Reduced." Readings below 400 m $\mu$  were not taken since  $\text{Na}_2\text{S}_2\text{O}_4$  itself absorbs in this region. If the curve thus obtained is now subtracted from that of the oxidized form, there is obtained the curve labeled "Difference" which is given in Fig. 1. This curve with a definite peak in the region of 440 to 450 m $\mu$  is suggestive of those given by various flavin compounds (*cf.* Ball (2)). If air is admitted to the reduced solution, the yellow color is restored, the density reading at 435 m $\mu$  returning to within 95 per cent of its original value. The reduction and oxidation process may be repeated indicating that a truly reversible oxidation-reduction system is involved in the color change.

The protein content of the solution employed in these spectrophotometric experiments was determined. The protein was denatured by heat and by the addition of alcohol and the precipitate obtained washed with water until free from ammonium sulfate as indicated by Nessler's reagent. The dry weight indicated 94.6 mg. of protein per ml. of solution. If we are dealing with a flavoprotein, as seems likely, then using the density readings given in Fig. 1 and

the dry weight, it is possible to calculate a minimum molecular weight. Employing Warburg's (10) value for riboflavin at  $445 \text{ m}\mu$  as  $\beta = 2.6 \times 10^7$  and the density reading of 0.35 at  $445 \text{ m}\mu$  for the curve labeled "Difference" in Fig. 1, it can be calculated that each milliliter of solution contains  $3.1 \times 10^{-8}$  mols of flavin. If the protein is homogeneous and contains one flavin group per molecule, then 94.6 mg. of protein must correspond to  $3.1 \times 10^{-8}$  mols. The minimum molecular weight of such a protein would, therefore, be three million.

The application of heat or addition of alcohol to a solution of the protein causes the formation of a white precipitate of denatured protein and leaves a

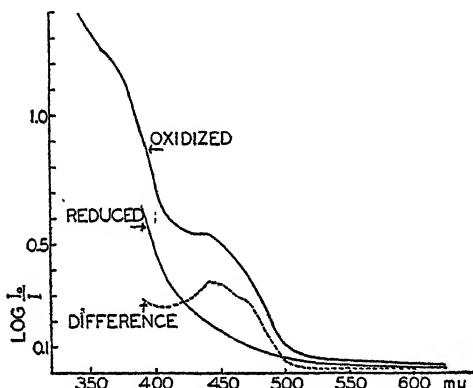


FIG. 1. The absorption spectra of the yeast yellow protein in the oxidized and reduced forms. Values are for a 1 cm. cell and for a solution containing 94.6 mg. of protein per ml.

clear yellow supernatant. The absorption spectra in the near ultraviolet of such supernatants vary according to the procedure employed for denaturation. Solutions obtained by the addition of 4 volumes of absolute ethyl alcohol to one volume of protein solution show much more absorption in the near ultraviolet than those obtained by heat denaturation. Control experiments show that the alcohol is not contributing to this extra absorption. The absorption spectrum of an alcoholic solution of the prosthetic groups resembles that given in Fig. 1 for the protein itself for the region 350 to 550  $\text{m}\mu$ . These observations suggested the presence of two prosthetic groups in the protein, one yellow in color and the other absorbing primarily in the ultraviolet. The following experiments substantiate this suggestion.

An alcoholic solution of the prosthetic groups of the protein was prepared as described above. This was evaporated to dryness on a steam bath and the small yellow residue resulting was taken up in water. The absorption spectrum of this solution is shown in Fig. 2, as curve 1. It resembles that of the protein

itself. This aqueous solution was now made acid to Congo red paper and extracted successively with small portions of  $\text{CHCl}_3$ . The clear colorless  $\text{CHCl}_3$  extracts were pooled, and mixed, and the absorption spectra of the chloroform extract and the clear yellow aqueous residue were measured. In Fig. 2, curve 2 is the spectrum of the aqueous yellow residue. It shows a more pronounced peak in the region of  $440 \text{ m}\mu$  which is characteristic of the flavins than does the original solution. The yellow color of this solution was discharged by the addi-

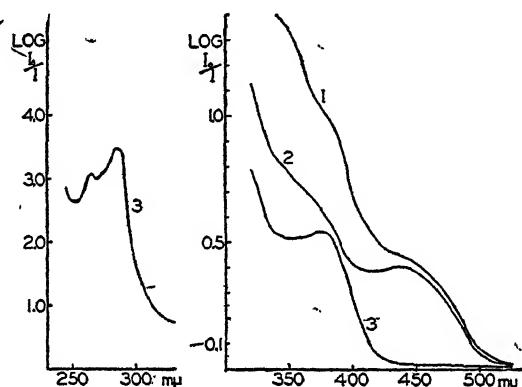


FIG. 2. The absorption spectra of the prosthetic groups of the yeast yellow protein.

Curve 1. An aqueous solution of the prosthetic groups obtained by alcohol denaturation of the protein. Curve 2. Aqueous residue of the solution shown in curve 1 after its extraction with  $\text{CHCl}_3$ . Curve 3.  $\text{CHCl}_3$  extract of solution shown in curve 1.

Final volume of  $\text{CHCl}_3$  solution equal to that of original aqueous solution of curve 1, thus curve 2 + curve 3 = curve 1.

All solutions were measured in a 1 cm. cell and contained per milliliter the amount of material obtained from 151 mg. of protein. See text for further details.

tion of  $\text{Na}_2\text{S}_2\text{O}_4$  and returned on shaking with air. Ascorbic acid did not cause such a reduction which is to be expected if the yellow color is due to a flavin. The chloroform solution has the spectrum shown by curve 3 in Fig. 2. It shows three peaks located at 375, 285, and 265  $\text{m}\mu$  with much stronger absorption at the latter two points. There is thus no doubt that two different compounds are present.

Further proof was now sought for the flavin nature of the yellow prosthetic group by submitting it to the procedure for production of a lumiflavin derivative. The yellow aqueous residue from the chloroform extraction was further treated with chloroform until little ultraviolet-absorbing material was extracted. It was then made 0.05 in  $\text{NaOH}$  and irradiated with white light for 3 hours with

cooling under running tap water. The irradiated yellow solution was again made acid to Congo red. Extraction with one portion of chloroform now removed practically all the yellow color from the aqueous residue. The chloroform solution so obtained had the characteristic greenish yellow fluorescence displayed by lumiflavin solutions. The absorption spectrum of the solution left little doubt that the yellow compound was flavin in nature. It showed two main peaks centered at 450 and 345 m $\mu$  with the peak at 450 m $\mu$  possessing on either side shoulders characteristic of lumiflavin (*cf.* Warburg (10)). The spectrum differed from that of lumiflavin in that the peak at 345 m $\mu$  was nearly the same height as that at 450 m $\mu$  but this may well be due to the presence of a residue of the other prosthetic group which absorbs in this region.

All the evidence thus points to the conclusion that the yellow color of the protein is due primarily to the presence of a flavin compound. Two flavin prosthetic groups are known, riboflavin phosphate and flavin-adenine dinucleotide. The presence of the latter compound can be detected by its ability to function as the coenzyme of the *d*-amino acid oxidase system (*cf.* (11)). Solutions of the yellow prosthetic group obtained from the yeast yellow protein by any of the described procedures did not act as a coenzyme for *d*-amino acid oxidase, nor did they prevent the functioning of added flavin-adenine dinucleotide in this test system. It would appear, therefore, that the flavin described here is either riboflavin phosphate or some unknown compound.

The nature of the prosthetic group extracted from acid solution by CHCl<sub>3</sub> is not known. It would appear to possess some group capable of undergoing dissociation, since it cannot be extracted from alkaline solutions by CHCl<sub>3</sub>. Aqueous solutions of the material may be obtained by solution of the faintly yellow residue left after evaporation of CHCl<sub>3</sub> solutions. The residue, however, dissolves more readily in weakly alkaline solutions than in water to give a faint but definitely yellow solution. This yellow color is not discharged by the addition of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> but it fades markedly on acidification of the solution. This change in color with pH is readily reversible and is reflected in a difference in the absorption spectra of the acid and alkaline forms. The acid aqueous form possesses a spectrum similar to that given by the CHCl<sub>3</sub> solution of this group shown in curve 3, Fig. 2. It possesses peaks at 260 to 265, and 285 m $\mu$  and shows general absorption in the region of 325 to 450 m $\mu$ . The alkaline aqueous form possesses one peak at 260 m $\mu$  which is higher than either of those for the acid form in this region, and shows general absorption extending from 325 to 500 m $\mu$ .

As yet, no catalytic function has been found for this flavoprotein. It has been tested manometrically for activity in the oxidation of the following substrates: glucose, hypoxanthine, acetaldehyde, sodium lactate, sodium succinate, *dl*-alanine, *L*-leucine, and glycine. It also failed to catalyze the reaction between reduced diphosphopyridine nucleotide and methylene blue.

## DISCUSSION

Green, Knox, and Stumpf (6) have also described the preparation of a flavoprotein from yeast for which no catalytic function could be found. Their compound does not appear to be the same as the one described here, though there is some resemblance in the absorption spectra of the two compounds. The flavoprotein obtained by Green *et al.* contains flavin-adenine dinucleotide and is brownish yellow in color. Moreover, the second prosthetic group which it contains does not appear to be released by denaturation of its protein carrier.

It is of interest to note that more and more flavoproteins with atypical absorption spectra and thus containing presumably two or more prosthetic groups are being discovered. In this group, in addition to the two yeast compounds mentioned above, may be listed xanthine oxidase (3), liver aldehyde oxidase (5), and the glucose oxidase obtained from *Penicillium notatum* (4, 9). The significance of this coupling of the flavin oxidation-reduction system with another prosthetic group in presumably the same protein molecule remains obscure. Indeed, the nature of the material present in addition to flavin in any one of these proteins is as yet unknown. One may presume that a vitamin is involved. In the case of the unknown prosthetic group of the flavoprotein described here, a comparison of its absorption spectrum with that of different vitamins was made. Some similarity is to be seen between its spectrum and that of folic acid (1) and vitamin B<sub>C</sub> (8). These substances, however, exhibit a change in spectra with pH which is just the reverse of that observed for the compound described here.

## SUMMARY

The yeast yellow protein contains two prosthetic groups, one of which is undoubtedly a flavin. The nature of the other is unknown, though some of its properties are described.

No catalytic function for this protein has as yet been found.

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## CULTURE CONDITIONS AND THE DEVELOPMENT OF THE PHOTOSYNTHETIC MECHANISM

### III. INFLUENCE OF LIGHT INTENSITY ON CELLULAR CHARACTERISTICS OF CHLORELLA\*

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Experimental material for studies on the photosynthetic process has been grown under a wide range of conditions. In the past little attention has been paid to differences in culture conditions since the photosynthetic mechanism was regarded as rigid and clearly defined. More recent work suggests that the various parts of the photosynthetic mechanism are distinct and separable. A possibility which now assumes added significance is that a range of experimental material with different photosynthetic characteristics may be obtained by control of previous conditions of growth. This would be a great aid to biochemical analysis of the mechanism.

A serious problem in any comparison of cells grown under different conditions lies in the selection of a reliable index of protoplasmic material to which metabolic rates may be referred. In previous studies on photosynthesis attempts express "absolute" rates have been limited in scope. Common procedure has been to compare the behavior of replicate samples of a single batch of cells. In metabolic studies on the algae and other microorganisms rates of metabolic processes have been expressed variously in terms of unit cell volume, unit dry weight, unit cell number, unit cell nitrogen, or unit chlorophyll. The present paper presents data describing the relationships between these different units of protoplasmic material for cells of *Chlorella pyrenoidosa* cultured under different conditions of light intensity. This study of effects of a single variable in culture conditions depends upon the use of an apparatus for the continuous culture of microorganisms described by Myers and Clark (1944).

#### EXPERIMENTAL

*Chlorella pyrenoidosa* (Emerson's strain) was grown in a modified Knop's solution in three units of the continuous-culture apparatus previously described. The Knop's solution contained 0.010 M MgSO<sub>4</sub>, 0.012 M KNO<sub>3</sub>, 0.009 M KH<sub>2</sub>PO<sub>4</sub>, 13.3 × 10<sup>-5</sup> M Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>, 56.0 × 10<sup>-5</sup> M sodium citrate, and 1.0 ml./liter each of the A<sub>5</sub> and B<sub>6</sub> solutions of Arnon (1938) which provide 0.5 parts per million B, 0.5 ppm. Mn, 0.05 ppm.

\* Supported by a grant from the University of Texas Research Institute.

Zn, 0.02 ppm. Cu, and 0.01 ppm. each of Mo, V, Cr, Ni, Co, W, and Ti. The medium was prepared as described in a previous paper (1944) and adjusted to a pH of 5.0.

Constant temperature in the chambers was provided by rapid circulation of water from baths maintained at 25.00° and 25.05° ± 0.05°C. Illumination was provided by four pair of 40 watt clear lumiline tungsten filament bulbs mounted end-to-end on four vertical holders spaced symmetrically about each chamber. Lamps rated at 120 volts were operated at 115 volts ± 1 volt as secured through a voltage stabilizer. Light intensity was adjusted by variation of the distance of the lamps from the chamber and by use of 16 mesh galvanized iron screens placed around the chamber or lamps or both. One layer of screen has a transmission of 66 per cent; two layers, of 43 per cent. To obtain the lowest intensities used it was necessary to substitute three 10 watt, 120 volt, S14 bulbs mounted on each of the four vertical holders. By placing these 25 cm. away a uniform illumination on the chamber could be secured. Measurements of light intensity were made with a Weston No. 603 foot-candle meter which has a photronic cell with quartz window.<sup>1</sup>

Sterile precautions of culture were maintained throughout and checked by daily samples of ~1 ml. withdrawn into sterile flasks of glucose-peptone broth. Upon any evidence of contamination cultures were discontinued.

In the continuous-culture apparatus a photometric device dilutes the culture suspension at such a rate that the density of population is maintained approximately constant. In the present study the population densities were arbitrarily adjusted at about 0.6 to 0.7 c.mm. cells per ml. In the chamber the algal suspension is contained in a 5 mm. annulus between two glass tubes. A rough check on the transmissions of several suspensions was made by illuminating with a narrow focused beam of light in a 5 mm. absorption cell placed just in front of a large photronic cell. This arrangement gives an approximate summation of the directly transmitted and forward scattered light. A suspension of maximum chlorophyll content (grown at 10 f.-c.) gave 73 per cent "transmission" as compared with the cell filled with water; a suspension of minimum chlorophyll content (grown at 360 f.-c.) gave 89 per cent "transmission." In the chamber an algal cell which is momentarily on the inside of the annulus gets

<sup>1</sup> Our experience with the calibration of the instrument should be cited. After a period of use of about 1 year the foot-candle meter was checked against a Bureau of Standards lamp operated through a carefully calibrated ammeter and found to read about 35 per cent high. It was returned to the manufacturer who corroborated the high calibration and inserted a new photocell unit and calibration. On return (and at the beginning of the experiments here reported) the instrument read about 4 per cent high against two different Bureau of Standards lamps; 6 months later (at the end of these experiments) it read 10 per cent low. Light intensity values reported here are meter readings without corrections which probably would not be significant. No accurate means of checking the linearity of the instrument were available. However, linear response with low resistance meters is a well known characteristic of this type of photocell.

much better than the above fractions of the incident flux since it is also exposed to the flux transmitted across the circle from the other side of the annulus. Because of the problems of scattering and the annular nature of the chamber the situation does not submit to precise analysis. However, it is clear that uniformity of illumination is good. Furthermore, the design of the apparatus insures that the effective light intensity is not changed by the growth or sampling of a culture. All factors considered, it is probably safe to estimate that no cell of a given suspension ever experiences a variation of light intensity of more than about 20 per cent.

Cells harvested from the apparatus have been examined for cellular characteristics. Parallel measurements of photosynthetic activity are reported in the following paper of the series.

Cell volume (cubic millimeters of packed cells per milliliter of suspension) was estimated by a centrifuging technique which in our hands has yielded good precision ( $\pm 2$  per cent). Duplicate 20 or 25 ml. aliquots of suspension are centrifuged out in conical centrifuge tubes of 50 ml. capacity cut down so that the capillary of a Van Allen thrombocytocrit (Van Allen, 1926) may be inserted to the bottom. The packed cells, resuspended in a little fresh Knop's solution of the same pH as the harvested suspension (pH 5.8), are sucked up into the bulb of the thrombocytocrit. A second small portion of the Knop's is used as a wash and also drawn up. The usual clip is placed over the thrombocytocrit capillary and the tube and duplicate centrifuged for 1 hour at 3300 r.p.m., 20 cm. radius, or a relative centrifugal force of 2420. As noted by others (Tang and French, 1933; Sargent, 1940) readings of the volume of packed cells approach a minimum value asymptotically. A minimum value is normally attained in less than 45 minutes. A 2nd hour of centrifuging produces less than 1 per cent decrease in reading. The reading remains unaltered for some time. The cells are tightly packed and at first can be expelled only by insertion of a wire. In time they loosen up and can be blown out. The capillary has a capacity of 30 c.mm. calibrated to 0.5 c.mm. and readable to 0.1 c.mm. Checks with weighed amounts of mercury show an accuracy of calibration of  $\pm 2$  per cent in a series of six tubes. Other checks show that the cell volume so determined is independent of the choice of aliquot size.

As an index of cellular material the cell volume has been widely used as an approximate measure and variously criticized as an exact measure in photosynthesis work (*cf.* Crozier, Tang, and French, 1934; Sargent, 1940; Tang and French, 1933; van Hille, 1938). The cell volume obtained in our hands by the above technique is a rapid and precise estimate of cellular material. It requires a total time of about 75 minutes and a working time of only about 15 minutes. The cell volume per milliliter of suspension was therefore run as a routine and used as a common denominator for other measurements which could not all be made simultaneously.

Cell numbers were estimated by hemocytometer counts of about 1000 cells in a total of four drops of suspension as previously described (Myers, 1944).

Cell dry weights were obtained by centrifuging known aliquots of suspension, washing twice in distilled water, and transferring the cells in a little water to tared weighing bottles. Cells were dried 24 hours at 105°C. As a check on the method of drying, several duplicate samples were dried at room temperature *in vacuo* and at 105°C. The dry weights obtained by the two methods were not significantly different though the dried cells were quite different in appearance. Cells dried at 105°C. gave a hard, coherent film from which they could not be readily suspended in water; those dried *in vacuo* formed a light, fluffy powder, easily resuspended.

Cell nitrogen was determined by slight modification of the method of Johnson (1941). Samples of suspension of 1 or 2 ml. were centrifuged out in test tubes and washed twice in water. Kjeldahl digestion and nesslerization were performed in the same tube. Nitrogen was estimated with a photoelectric colorimeter employing Cornell filters No. 3387 and No. 5030 and calibrated with a standard curve. In our hands the precision of the method is estimated at  $\pm 5$  per cent.

Total chlorophyll was determined by extracting duplicate centrifuged samples (4 to 10 ml. of suspension) with boiling methanol, diluting to 10.0 ml., and reading in a photoelectric colorimeter employing a 1 cm. water cell, a sealed 1 cm. cell of  $M/3$  copper sulfate, and an RG5 Jena filter. This gives a band extending from 640 to 710  $m\mu$  with a peak at 660  $m\mu$ . A calibration curve in relative units was obtained by quantitative dilution of a cell extract. Data are reported in these relative "chlorophyll units."<sup>2</sup> Inadequacies of the method (use of hot methanol, the broad wave length band of our colorimeter) are fully realized. However, as a relative estimate of total chlorophyll, it is rapid and reasonably precise ( $\pm 2$  per cent).

In practice samples of the culture suspensions were harvested at 24 hour (or other) intervals, leaving as an inoculum for the next interval a constant amount of suspension as indicated by a mark at an arbitrary height on the chamber. When a constant density of population is being maintained the relation between the amount of the inoculum and the amount of the sample is an index of rate of growth. From the characteristics of the continuous-culture apparatus perfectly logarithmic growth is to be expected, regardless of the population density maintained. Rate of growth is expressible in terms of the constant  $k$  in the equation  $\log \frac{N}{N_0} = k t$ . In applying the equation,  $N_0$  is expressed in milliliters of inoculum at the beginning of each interval,  $N$  in total milliliters of

<sup>2</sup> A check against a chlorophyll preparation (American Chlorophyll Company, 5 $\times$ ) gave the result that 1 unit = 3.77 micrograms of chlorophyll. This would give chlorophyll analyses ranging from 4 to 20 per cent of the dry weight. In view of the values of 2 to 7 per cent reported by Sargent (1940) and calculated approximately from the data of Emerson and Arnold (1932) our values seem unreasonably high and we hesitate to report chlorophyll in absolute units. Cellular extracts and a methanol solution of the commercial preparation give nearly identical red absorption bands as checked by a Beckman spectrophotometer. A likely source of error not investigated is the purity of the commercial chlorophyll preparation.

suspension (milliliters of inoculum + milliliters of sample) at the end of a unit time interval,  $t$ , of 1 day.

### RESULTS

The data are listed in Table I and presented graphically in Fig. 1. Each datum is an average of three determinations made on different days (in some cases weeks apart) over a period in which population density and growth rate were essentially constant. Population densities are presented as evidence that this is not a variable in the present experiments. Some experiments were run simultaneously at different light intensities; the general chronological order of

TABLE I

Light intensity f.-c.	Relative growth rate $k^*$	Dry weight $gm. \times 10^{-6}/$ $c.mm.$	Cell nitrogen $\gamma/c.mm.$	Cell No. $\times 10^8$ cells/ $c.mm.$	Cell chlorophyll $units/c.mm.$	Population density $c.mm./ml.$
360 (5)	0.840	27.0	20.3	6.3	2.7	0.60
92 (3)	0.830	24.2	21.9	13.2	5.3	0.70
55 (4)	0.730	23.7	20.7	15.3	6.9	0.70
35 (1)	0.576	22.2	21.3	26.0	9.0	0.64
19 (2)	0.336	—	—	—	—	—
(6)	0.362	—	—	24.0	10.0	0.69
10 (7)	0.180	21.4	21.5	31.2	11.6	0.65
6 (8)	0.103	—	—	28.2	12.7	0.70

$$* k = 1/t \times \log \frac{N}{N_0}, t \text{ being a time unit of 1 day.}$$

experiments is indicated by the figures in parentheses after the light intensities in Table I.

The light intensity curve of growth in Fig. 1 is a typical light saturation curve and will be discussed in this connection in the following paper. It is presented here only for comparative purposes.

A marked feature of the curves is the concomitant variation in cell number and chlorophyll per unit volume of cells. With higher light intensities of culture the cells become increasingly larger but with about the same chlorophyll content per cell. This is an entirely unexpected phenomenon. It appears that the adjustment of cell size and chlorophyll content are correlated by a cellular mechanism in such a way that there are always about the same number of chlorophyll molecules per cell. The question now arises whether there are any real changes in chloroplast size and structure that accompany the variation of chlorophyll concentration in terms of cell volume. This has important implications in relation to theories of the *photosynthetic unit*.

The intermittent light studies of Emerson and Arnold (1932) revealed that

for very short intense flashes separated by comparatively long dark periods the photosynthetic yield per flash per cubic millimeter of cells is an approximately linear function of the chlorophyll content per cubic millimeter of cells. The relationship was such as to indicate about 2500 chlorophyll molecules per molecule of carbon dioxide reduced per flash. This observation gave rise to the concept of a *photosynthetic unit*, a physical unit in which a large number of chlorophyll molecules somehow participate in the reduction of one molecule of carbon

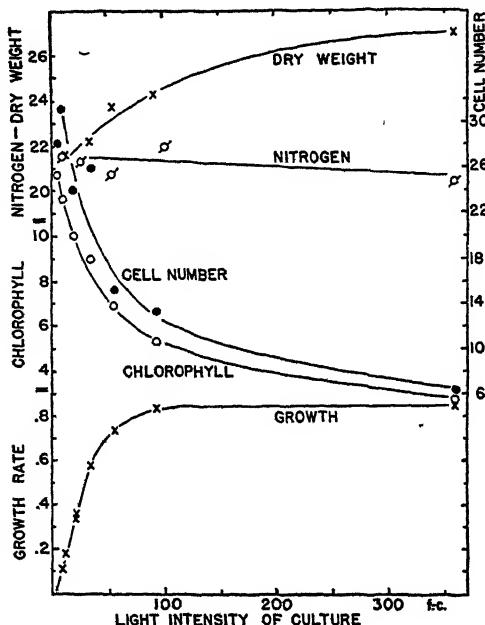


FIG. 1. Growth rate and cellular characteristics (cellular quantities/unit cell volume) as a function of light intensity of culture. Units are  $10^{-5}$  gm. dry weight/c.mm., micrograms nitrogen/c.mm.,  $10^6$  cells/c.mm., arbitrary units chlorophyll/c.mm.

dioxide. Alternative explanations have been proposed (*cf.* Emerson, 1937) and objections to the original theory have been made on various grounds. Our data offer a new point of view in interpretation of the observations of Emerson and Arnold.

When chlorophyll concentration is varied by light intensity of culture it appears that the effect is caused almost entirely by variation in cell size without much change in number of chlorophyll molecules per cell. It seems quite likely that a similar phenomenon occurs when chlorophyll is varied by the use of mercury or neon lamps as used by Emerson and Arnold. At least part of the difference between mercury and neon light must lie in a variation in effective intensity. Neon produced cultures which grew more rapidly and yielded cells

with lower chlorophyll concentration so that it seems comparable with our use of high intensity. If it be assumed that the same parallel between chlorophyll concentration and cell number occurred in the experiments of Emerson and Arnold as in ours, then carbon dioxide reduced per flash per cell (or per chloroplast) also becomes a constant value. In effect, the argument suggests a cellular or physiological type of the *photosynthetic unit*, which has quite different implications from those required of a physical type of unit.

Data on chlorophyll vary with light intensity in qualitatively the same fashion as previously reported for *Chlorella*. Sargent (1940) found chlorophyll contents of 3.3 per cent and 6.6 per cent respectively for cells grown under "high" and "low" light intensities. As a means of systematically varying chlorophyll concentration the fivefold range obtained here by control of light intensity may be compared with the sixfold range obtained by Emerson (1929) by control of iron concentration in *C. vulgaris*, the ten- to twelvefold range obtained by Fleischer (1934) by control of iron and nitrogen concentrations in the Cornell strain of *Chlorella*, and the sevenfold range obtained by Emerson and Arnold (1932) by use of illumination from mercury and neon gaseous discharge tubes for *C. pyrenoidosa*.

The dry weight per unit volume of cells changes more slowly with light intensity of culture, the total variation amounting only to about 20 per cent in the range of intensities studied here. Apparently cells grown at higher light intensities store less hydrated materials. In terms of dry weight and cell number our values are consistent with those cited by Tang and French (1933) for the same strain of *Chlorella*. Their data give 1 c. mm. cells =  $18.7 \times 10^6$  cells =  $23.2 \times 10^{-5}$  gm.<sup>3</sup> Both of their values fit in between our values for cells cultured at 55 and 35 f.-c.

The variation in nitrogen content per unit volume is small and probably not significant. The nitrogen content of the cells is surprisingly high (7.5 to 10 per cent of the dry weight). Check analyses by the Dumas method<sup>4</sup> on a sample of dried cells grown at 92 f.-c. yielded 8.3 per cent nitrogen as compared to 9.1 per cent calculated from the appropriate values of Table I. The latter calculated value is an indirect one since the nitrogen and dry weight were determined on separate aliquots and would be higher than a direct determination on the dry material if any nitrogenous compound is lost on drying. The possibility of loss of ammonia on drying was tested by evacuating, through a trap of dilute acid, a tube containing a considerable quantity of cells. Subsequent nesslerization revealed no ammonia in the acid trap. We are unable to explain the somewhat higher value obtained by our micro Kjedahl procedure than that obtained by

<sup>3</sup> The last value is corrected in its decimal point according to a personal communication from Dr. C. S. French.

<sup>4</sup> We are indebted to Dr. J. W. Melton of the Department of Chemistry for these analyses.

Dumas analysis. At any rate the nitrogen content of *Chlorella* is high and in the same range as the nitrogen content of 5.9 per cent to 9.6 per cent found for isolated chloroplasts of several higher plants by Menke (1938) and Neish (1939).

#### DISCUSSION

The results do not in themselves dictate the selection of any one particular cellular quantity as an index for metabolic activity in *C. pyrenoidosa*. Cell nitrogen has frequently been chosen on the theory that it provides the best measure of cellular machinery. Unfortunately the nitrogen analysis is too slow and (in our hands) of too low a precision for routine work. It closely parallels cell volume. This is not surprising if it be considered that the hydration of the cell is particularly influenced by its protein content. There is nothing to indicate any marked changes or instability in the water content of the cells. We have selected cell volume as a convenient index of cellular material in investigating the photosynthetic characteristics of cells grown under different light intensities. The data presented relate other cellular characteristics to cell volume so that metabolic rates may be converted to other indices at will.

#### SUMMARY

1. *Chlorella pyrenoidosa* has been grown in a continuous-culture apparatus under various light intensities provided by incandescent lamps, other conditions of culture being maintained constant. The harvested cells were analyzed for cell number, dry weight, nitrogen, and chlorophyll per unit cell volume.
2. Cell nitrogen and cell volume are parallel measures of cellular material over the range of light intensity studied.
3. The dry weight per cell volume increases slowly with light intensity, showing about a 20 per cent variation.
4. Chlorophyll concentration and cell number show a concomitant decrease with increasing light intensity, varying in such a way that there are always about the same number of chlorophyll molecules per cell. It is considered that this phenomenon has bearing on the interpretation of data which has led to the theory of the photosynthetic unit.

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## CULTURE CONDITIONS AND THE DEVELOPMENT OF THE PHOTOSYNTHETIC MECHANISM

### IV. INFLUENCE OF LIGHT INTENSITY ON PHOTOSYNTHETIC CHARACTERISTICS OF CHLORELLA\*

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Numerous data in the literature indicate that the photosynthetic characteristics of an organism depend upon previous conditions of culture (e.g. Aufdem-Garten, 1939; Emerson, Green, and Webb, 1940; McAlister and Myers, 1940; Myers and Burr, 1940; Sargent, 1940). The apparatus for the continuous culture of unicellular algae described by Myers and Clark (1944) permits the systematic study of the effects of culture conditions by examination of a single variable at a time. The present paper considers the effect of light intensity of culture on the subsequent photosynthetic behavior of *Chlorella pyrenoidosa*. Supporting data on parallel effects of light intensity on cellular characteristics are presented in the preceding paper of the series (Myers, 1946).

#### EXPERIMENTAL

*Chlorella pyrenoidosa* was grown at 25°C. with 4.4 per cent carbon dioxide in Knop's solution in three units of the continuous-culture apparatus as described in the preceding papers (1944, 1946). The data to be reported were obtained in two series of experiments. Series I was done before and Series II in parallel with the work of the preceding paper.

*Series I.*—Photosynthetic characteristics were studied manometrically by the Warburg technique using a bath with glass bottom thermostated at  $25 \pm 0.05^\circ\text{C}$ . Some measurements were made in a Knop's solution (pH 4.45) saturated with 4.4 per cent carbon dioxide. An aliquot of cells was centrifuged out, washed in Knop's, and then taken up in a measured volume of fresh Knop's solution. Equal amounts of this final suspension were pipetted into each of a pair of vessels and an additional measured volume of Knop's added to one of the vessels to obtain different liquid:gas ratios. The rates of O<sub>2</sub>-evolution and CO<sub>2</sub> uptake could then be calculated. Other measurements of photosynthesis were made in the Warburg No. 11 buffer (0.005 M K<sub>2</sub>CO<sub>3</sub> + 0.095 M KHCO<sub>3</sub>) using a suspension prepared by centrifuging and washing an aliquot of cells in water and in buffer and then taking up in a measured volume of buffer. In all cases the rate in terms of millimeters of pressure change per hour was determined graphically by taking 5 minute readings over a period of about 1 hour. The period of illumination was followed by a period of about an hour in darkness for measurement of respiration.

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Illumination for the measurements was provided by a bank of seventeen 60 watt Mazda lamps closely arranged in two rows 27 cm. below the reaction vessels. Lamps rated at 120 volts were operated at  $112 \pm 1$  volt as provided by a voltage stabilizer and variable transformer. About 600 foot-candles of illumination are provided at the positions of the reaction vessels. In determination of the light intensity curves, the rates in five vessels were measured simultaneously. Intensity was then varied by the use of Jena NG-series neutral filters attached to the bottoms of the vessels by holders which also masked out all stray light. The net transmission of the filters was estimated from the spectral absorption curves obtained on a General Electric recording spectrophotometer. The incident intensity upon the five filter vessel combinations was not uniform but was measured each day by a calibrated barrier type photocell enclosed in an Erlenmeyer flask and immersed in the bath. By a slide and carriage arrangement the photocell could be moved to the mean position of each vessel.

In practice the culture suspension was harvested at daily intervals, leaving as an inoculum for the next interval a constant amount as indicated by a mark at an arbitrary height on the chamber. The relation between the amount of inoculum and the amount of the daily sample is an index of rate of growth. A culture was maintained at a given light intensity until the rate of growth and the rate of photosynthesis became constant. Thereafter, the light intensity curves in No. 11 buffer and measurements under 4 per cent CO<sub>2</sub> were made as described above.

The population densities (cubic millimeters of cells per milliliter of suspension) were determined on duplicate aliquots of suspension by centrifuging in Bauer and Schenk tubes as described in the second paper of this series (Myers and Clark, 1944). Densities of population were arbitrarily adjusted to about 1.0 c.mm. per ml. (actually ranging from 0.90 to 1.64 in different cultures). In each experiment several determinations of pH were made on the freshly harvested suspension by means of a Coleman glass electrode.

*Series II.*—Data obtained in the first series (see below) indicated that the assimilatory quotient does not vary systematically with light intensity of culture and averages about -0.92. The manometric technique was therefore modified by making all measurements in Knop's solution saturated with 4.4 per cent carbon dioxide, using single flasks for each determination, and assuming an assimilatory quotient of -0.90. Light intensity curves were obtained as a routine in five flasks as otherwise described in Series I. Cell volumes were determined on duplicate aliquots by centrifuging in Van Allen thrombocytocrit tubes as described in the preceding paper (1946). Population densities were adjusted to lower values (0.60 to 0.70 c.mm. cells/ml.) since other data (yet to be published) indicate that at low light intensities rate of growth is somewhat higher at lower population densities at which there is less mutual shading.

#### RESULTS

Light intensity curves of Series I obtained with cells immersed in the No. 11 carbonate-bicarbonate buffer are presented in Figs. 1, 2, and 3. Abscissas represent light intensity in foot-candles during the measurements. Ordinates

represent rate of  $O_2$  evolution (in c. mm.  $O_2$ /hour/c.mm. cells) corrected for respiration. Although respiration rates were not determined with very great precision they seemed to depend somewhat on the light intensity of culture.

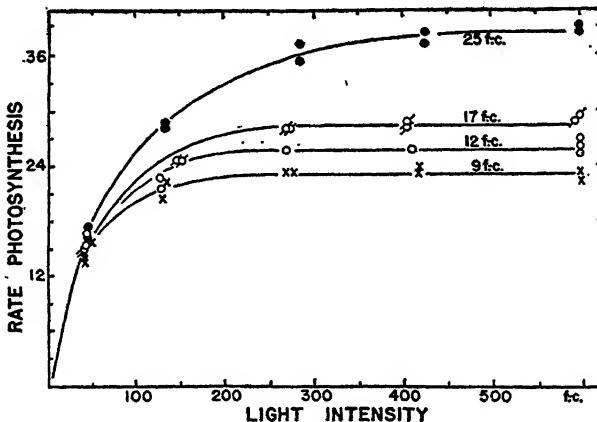


FIG. 1. Light intensity curves for cells cultured at the light intensity indicated on each curve. Rate = c.mm.  $O_2$ /hour/c.mm. cells measured in No. 11 buffer. Series I.

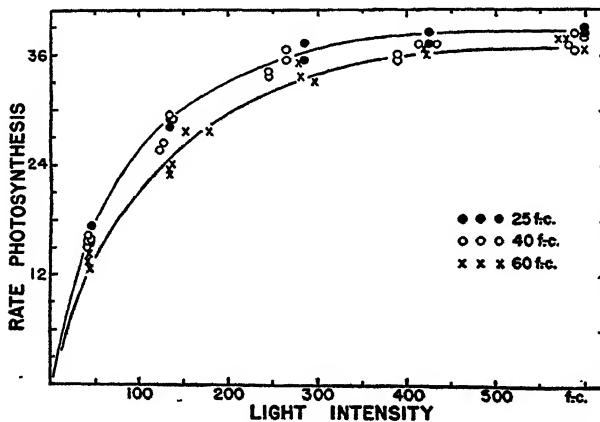


FIG. 2. Light intensity curves for cells cultured at the light intensity indicated for the various points. Rate = c.mm.  $O_2$ /hour/c.mm. cells measured in No. 11 buffer. Series I.

Cultures grown at higher intensities (Fig. 3) gave respiratory rates in the range of 1.8 to 2.4 c.mm.  $O_2$ /hour/c.mm. cells; at the lower intensities (Fig. 1), 1.2 to 1.8.

Each of the curves of Figs. 1, 2, and 3 characterizes cells grown at the indicated light intensity. Each curve is drawn as a best fit for experimental points

obtained on at least two different samples from the same culture. (For purposes of comparison the curves for cells grown at 25 and 60 f.-c. are repeated in different figures.) In terms of their response to light intensity cells grown at different intensities differ in two important respects: (1) the maximum rate of photosynthesis attained at high intensities and (2) the shape of the light intensity curve at lower intensities.

The maximum rates of photosynthesis attained for each of the curves of Figs. 1, 2, and 3 are plotted against the light intensity of culture in Fig. 4 *b*. Three regions of light intensity, AB, BC, CD, can be recognized in Fig. 4 *b* corresponding to the groupings of the curves in Figs. 1, 2, and 3, respectively. Maximum capacity for photosynthesis is attained by cells grown at intensities in the range of 25 to 60 f.-c. The exact nature of the region BC (whether tilted, or flat as drawn) cannot be decided. As indicated by Fig. 2, the precision of the measurements is hardly good enough to distinguish between 25, 40, or 60 f.-c. Below a light intensity of growth of about 25 f.-c. the maximum capacity for photosynthesis falls off rapidly (BA). Above about 60 f.-c. the maximum capacity for photosynthesis falls off slowly (CD).

Measurements of rates of photosynthesis in Knop's solution + 4 per cent CO<sub>2</sub> under 600 f.-c. (saturating light and CO<sub>2</sub>) yielded the data plotted in Fig. 4 *a*. No determinations of respiratory rates were made; the respiration corrections used (1.2 to 2.4 c.mm. O<sub>2</sub>/hour/c.mm. cells) were obtained from the measurements in the carbonate-bicarbonate buffer. Mean values of the assimilatory quotient for cells grown at the different light intensities are presented as the last column of Table I. Values of the quotient did not vary significantly with the possible exception of the lower value of -0.86 obtained for cells grown under 3.25 f.-c. The shape of the curve is similar to that of curve 4 *b*. It is of interest that for cells cultured at intensities above 25 f.-c. the rate of photosynthesis measured in Knop's + 4 per cent CO<sub>2</sub> is roughly 15 to 20 per cent higher than the rate obtainable in the No. 11 buffer (*cf.* Fig. 4 *a* and 4 *b*). Only for cells cultured at very low intensities do the two methods yield equivalent values.

That the curves 4 *a* and 4 *b* are not a chance result of the order of experiments or the particular population density or pH of each experiment can be seen by inspection of the first four columns of Table I. The light intensities used are listed in chronological order for each culture.

Presented in the fifth column of Table I are the relative growth rates for each light intensity.<sup>1</sup> Fig. 4 *c* shows the data on growth rate plotted against the

<sup>1</sup> In the continuous-culture apparatus, which insures a perfectly logarithmic growth, the relative growth rate, *k*, is defined by the equation  $\log \frac{N}{N_0} = k t$ . In applying the equation, *N*<sub>0</sub> is expressed in milliliters of inoculum at the beginning of each interval and *N* in terms of total milliliters of suspension (*N*<sub>0</sub> + milliliters sample) at the end of a time interval, *t*, of 1 day.

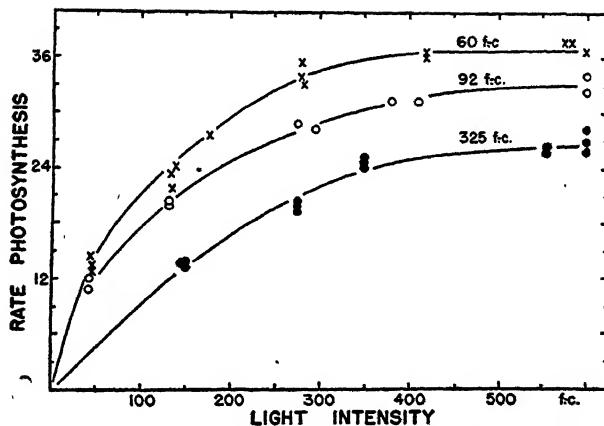


FIG. 3. Light intensity curves for cells cultured at the light intensity indicated on each curve. Rate = c.mm.  $O_2$ /hour/c.mm. cells measured in No. 11 buffer. Series I.

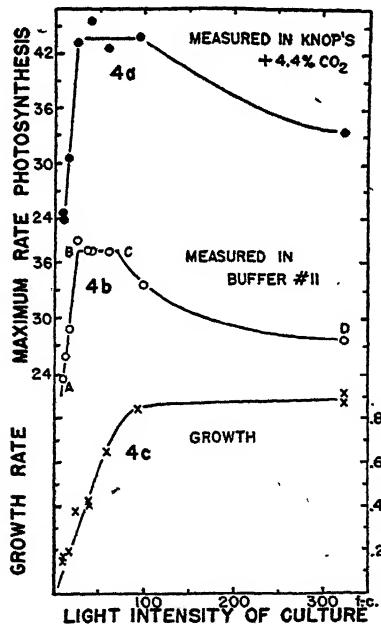


FIG. 4. (a) The maximum rate of photosynthesis (c.mm.  $O_2$ /hour/c.mm. cells) measured at 625 f.c. in Knop's + 4.4 per cent  $CO_2$  as a function of the light intensity of culture. (b) The maximum rate of photosynthesis (c.mm.  $O_2$ /hour/c.mm. cells) measured 625 f.c. in the Warburg No. 11 buffer as a function of the light intensity of culture. (c) Relative growth rate as a function of the light intensity of culture. Data of Series I.

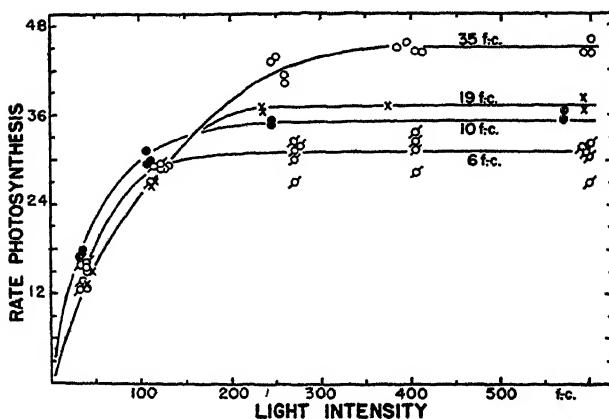


FIG. 5. Light intensity curves for cells cultured at the light intensity indicated on each curve. Rate = c.mm. O<sub>2</sub>/hour/c.mm. cells measured in Knop's + 4.4 per cent CO<sub>2</sub>. Series II.

TABLE I  
Culture Data

Culture No.	Culture intensity	Population density	pH	Growth rate (see text)	Assimilatory quotient (see text)
38	325	1.34	5.8	0.910	—
40	325	1.56-1.64	5.8	0.865	0.86
	95	0.92-1.08	5.8	0.840	0.91
	40	1.24	5.8	0.432	—
	60	1.16-1.24	5.9	0.648	0.89
41	40	0.94-1.02	5.8	0.408	0.94
	12	1.06-1.14	5.7	0.168	0.93
42	25	0.90-0.94	5.8	0.384	0.93
	9	1.18-1.20	6.0	0.144	0.93
43	17	1.18-1.22	5.9	0.192	0.94

light intensity of culture. At low intensities ( $< 60$  f.-c.) growth rate is proportional to light intensity. At high intensities ( $> 100$  f.-c.) growth is nearly independent of light intensity. A similar type of curve was obtained by Bristol-Roach (1928) from a study of rates of growth of *Scenedesmus*.

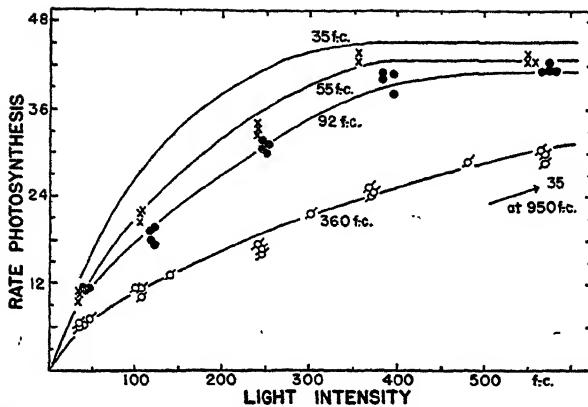


FIG. 6. Light intensity curves for cells cultured at the light intensity indicated on each curve. Rate = c.mm.  $O_2$ /hour/c.mm. cells measured in Knop's + 4.4 per cent  $CO_2$ . Series II.

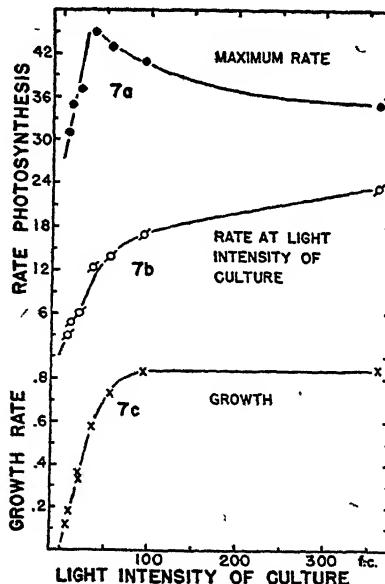


FIG. 7. (a) The maximum rate of photosynthesis (c.mm.  $O_2$ /hour/c.mm. cells) measured at 625 f.c. in Knop's + 4.4 per cent  $CO_2$  as a function of light intensity of culture. (b) The rate of photosynthesis (c.mm.  $O_2$ /hour/c.mm. cells) prevailing in the cultures for each light intensity of culture estimated from Figs. 5 and 6. (c) Relative growth rate as a function of light intensity of culture. Data of Series II.

The data of Series II experiments are presented in Figs. 5, 6, and 7. Light intensity curves obtained in Knop's solution + 4.4 per cent carbon dioxide follow the same general trend as those of the first series obtained in the carbonate-bicarbonate buffer. One difference appears in the actual crossing of the curves in Fig. 5 indicating for cells grown at the lower light intensities an enhanced photosynthetic ability measured at low intensities. This is a phenomenon commonly recorded in the literature of "sun" and "shade" plants (*cf.* Boysen-Jensen and Müller, 1929) but not observed by Sargent (1940) in his study on *Chlorella*. The appearance of the phenomenon here is not entirely consistent and it is difficult to see why a similar effect is not apparent in Fig. 1 of the first series. Another possible difference lies in the lack of any plateau in Fig. 7 *a* as compared to Fig. 4 *a*, although this hinges upon the significance of a single point of each curve at 92 to 95 f.-c.

The light intensity curve of growth (Fig. 7 *b*) of the second series is somewhat steeper than that of the first series (Fig. 4 *c*), probably reflecting the decreased population densities and better light distribution maintained in the second series. Both growth curves appear to extrapolate toward zero light intensity although this is obviously impossible since some energy must be required to maintain the basal metabolism of the culture.

#### DISCUSSION

Cells cultured within the range of light intensities reported here always develop a capacity for a maximum rate of photosynthesis<sup>2</sup> much higher than they ever experience during growth. This is indicated in Fig. 7 *b* which is drawn from data interpolated from the curves of Figs. 5 and 6 in order to express the rate actually attained in the culture chambers. At higher intensities of culture (> 35 f.-c.) the capacity for photosynthesis (Fig. 7 *a*) decreases although the rate actually attained during culture (Fig. 7 *b*) increases. Presumably at still higher intensities or culture (> 360 f.-c.) cells might be produced with a capacity for photosynthesis no greater than actually experienced during growth. (Like conclusions obtain from a similar analysis of the curves of Figs. 1, 2, and 3.)

The above argument rests on the assumption that the rates of oxygen evolution measured in the Warburg flasks approximate the rates actually experienced

<sup>2</sup> As used herein *capacity for photosynthesis* or *maximum rate of photosynthesis* describes the rate of photosynthesis, in terms of oxygen evolution, which obtains under conditions of light and carbon dioxide saturation at 25°C. According to classical interpretation it is determined by the rate of the *dark* or enzymatic or Blackman reactions. Actually any such measured rate is merely a rate of oxygen evolution and includes the effects of any processes opposing photosynthesis (*e.g.* photooxidations) over and above the dark respiration.

in the culture chambers. Several experiments on cells grown at higher light intensities have confirmed this assumption. Duplicate samples, one taken directly from the culture suspension, and the other with cells washed and resuspended in fresh Knop's solution of the same pH (5.8), have been compared in Warburg vessels of approximately the same volume. Retention of carbon dioxide by the fluid occurs at this pH so that rates of oxygen exchange cannot easily be calculated, but equal rates in vessels of the same volume and liquid: gas ratios must mean equal rates of oxygen evolution. Comparisons have also been made of cells washed and resuspended in fresh Knop's at pH 4.5 with cells in the original suspension adjusted to pH 4.5. Rates obtained for cells in the original suspension were generally slightly but not significantly higher than those obtained for washed cells in fresh medium. Fig. 7 b therefore seems a justifiable estimate of rate of photosynthesis attained in the culture chambers.

From Fig. 7 a it appears that at a rather narrow optimum range of light intensity of culture *C. pyrenoidosa* develops a maximum capacity for photosynthesis in terms of oxygen evolution per unit volume of cells. Above about 50 f.-c. there occurs a falling off in photosynthetic capacity with increasing intensity of culture. This appears to be correlated with the transition region between the light-limited and light-saturated rate of growth (Fig. 7 c).

The limitation of growth rate is in itself an interesting problem. Certainly the factor causing the limitation is not located within the photosynthetic mechanism since none of our light intensity curves of photosynthesis show complete light saturation in the cultures themselves. This is in accord with the data of Bristol-Roach (1928) who studied the effects of glucose and light intensity on rate of growth of *Scenedesmus*. At low light intensities the addition of 1 per cent glucose more than doubled the rate of growth; at high intensities it had practically no effect.

The data suggest the hypothesis that at light intensities above those which limit growth ( $\sim 50$  f.-c.) the cells develop mechanisms to dispose of the excess photosynthetic products above those used in the usual dissimilatory processes. One such mechanism might easily depend upon phooxidation processes which are known to occur (Franck and French, 1941; Myers and Burr, 1940), or upon "oxidation processes inside the assimilatory mechanism" in the sense of Gaffron (1939, 1940; cf. also Franck and Gaffron, 1941). In terms of such processes the falling off of curve 7 a and the bending over of curve 7 b above 50 f.-c. may be reasonably explained. An important implication of this line of reasoning is that (for cells as usually cultured) any photosynthesis-saturating light intensity is "unphysiological" in the sense that photosynthetic products must be accumulating faster than they can be handled by the usual cellular mechanisms that lead to growth. In long time experiments "effects of light on respiration" and other anomalies would seem to be expected.

Still another explanation must be found for the phenomenon illustrated by comparison of curves 7b and 7c. In changing from a light intensity of culture of 92 to 360 f.-c. the rate of growth is not increased; yet the net rate of oxygen evolution in the culture increases appreciably. At 360 f.-c. photosynthetic products are produced faster and must result in proportionately greater storage material or must diffuse out of the cells into the culture medium. Probably both of these effects occur. From the data of the preceding paper (1946)

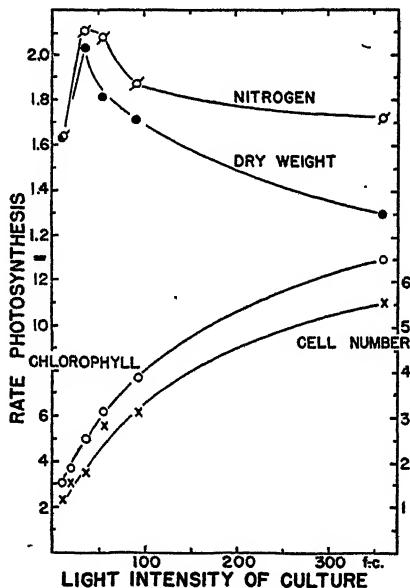


FIG. 8. Maximum rate of photosynthesis (c.mm.  $O_2$ /hour/unit cellular quantity) as a function of light intensity of culture for various indices of cell quantity. Data of Fig. 7a recalculated in terms of the data of Table I of the preceding paper (Myers, 1946). Units of cellular quantity are micrograms nitrogen,  $10^{-5}$  gm. dry weight, arbitrary units chlorophyll,  $10^6$  cells for cell number.

cells grown at 360 f.-c. have a 10 per cent greater dry weight than at 92 f.-c. At the same time the algae evidently secrete some organic material into the media since bacterial growth always develops in cultures (even without citrate) if sterile precautions are not maintained.

The falling off in capacity for photosynthesis at culture intensities below 25 f.-c. (Figs. 4a, 4b, and 7a) finds no apparent explanation. Inspection of the light intensity curves suggests that the effect observed here involves a quite different factor in the photosynthetic mechanism than that which operates at a high culture intensity (*cf.* Fig. 1 with Fig. 3 and Fig. 5 with Fig. 6). The

light intensity curves are of further interest in relation to those of previous workers. Cells cultured at the lowest intensities (Fig. 1) give curves approaching the Blackman type with a relatively short transition between the light-limiting and light-saturating portions of the curve. Unfortunately the true slopes of the curves under the light-limiting range of intensities cannot be estimated accurately from the present data although this is a matter of considerable importance.

The data of Fig. 7a have been replotted in Fig. 8 in terms of other indices of cell quantity obtained from Table I of the preceding paper (1946). The curve of Fig. 7a in terms of unit cell volume is not changed in shape if plotted in terms of unit dry weight or unit nitrogen. In each case maximum capacity for photosynthesis is attained at a light intensity of culture of about 35 f.-c. In terms of rate per cell or rate per unit chlorophyll, however, this effect is entirely masked by the pronounced increase in cell size and decrease in chlorophyll concentration with increasing light intensity of culture. In terms of unit chlorophyll or unit cell number the maximum capacity for photosynthesis increases continuously with increasing light intensities of culture.

As a means of varying the capacity for photosynthesis the control of light intensity as used here affords a possible variation of 30 to 45 per cent from the maximum value, which is approximately that obtained by Sargent (1940). It is much less than the 85 per cent variation recorded by Emerson and Arnold (1932) on cells grown under neon and mercury lamps. However, they acknowledged that they did not attain a maximum light-saturated rate for cells poor in chlorophyll which would make their range of variation appear greater than it actually was.

#### SUMMARY

1. *Chlorella pyrenoidosa* has been grown in a continuous-culture apparatus under various light intensities provided by incandescent lamps, other conditions of culture being maintained constant. Light intensity curves for cells immersed in the No. 11 Warburg buffer and in Knop's solution + 4.4 per cent CO<sub>2</sub> at a saturating light intensity were determined as characteristics of the photosynthetic mechanism. These characteristics were referred to the centrifuged cell volume as an index of quantity of cellular material.

2. Cells grown at intensities in the range of about 35 f.-c. develop a capacity for a high rate of photosynthesis (c.mm. O<sub>2</sub>/hour/c.mm. cells). At culture intensities above or below this range the cells produced have a lower capacity for photosynthesis. A similar effect is observed for rate of photosynthesis per unit dry weight or rate per unit cell nitrogen.

3. The rate of photosynthesis per cell or rate per unit chlorophyll shows no maximum at any light intensity of culture but increases continuously throughout the range of light intensities studied.

4. Maximum rate of growth is attained at a light intensity of about 100 f.-c. The hypothesis is advanced that at culture intensities above that needed to give maximum rate of growth (100 f.-c.) a mechanism is developed which opposes the photosynthetic process and removes the photosynthetic products.

5. The low capacity for photosynthesis shown by cells grown at culture intensities below 35 f.-c. finds no immediate explanation.

6. The shape of the light intensity curve is markedly affected by the light intensity at which the cells have been cultured. Cells grown at lower intensities give light intensity curves approaching the Blackman type with a short transitional region between light limitation and light saturation.

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# THE PENETRATION OF VESICANT VAPORS INTO HUMAN SKIN\*

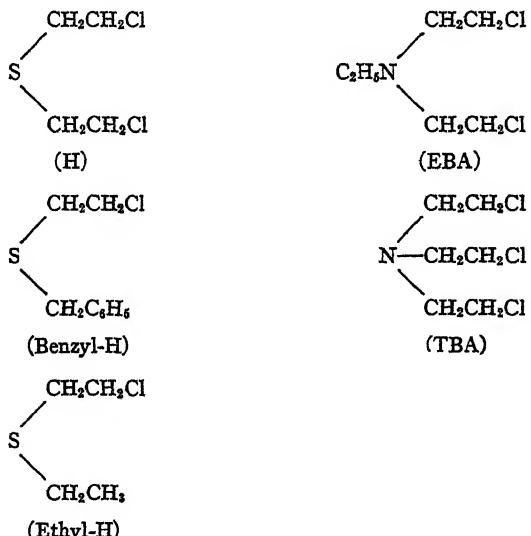
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Of obvious importance in the mechanism of action of vesicant agents are the relative rates at which different agents penetrate the human skin. The present study was undertaken to secure basic information on this question. Because of the experimental difficulties envisaged in a quantitative study of the penetration of vesicants as liquids, the present work was confined to the measurement of the penetration of vesicant vapors.

The problem has been resolved into four phases: (1) the development of precise analytical methods for measuring the amount of vesicant penetrated; (2) the construction of a suitable apparatus for applying vesicants, as vapors, to small areas of the skin; (3) a thorough test of the analytical methods and apparatus by means of appropriate model experiments; (4) the actual measurement of the rate of penetration of the vesicant vapors into human skin.



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† Died, November 7, 1944.

The following agents, the formulae of which are given above, were employed in this study: bis( $\beta$ -chloroethyl)sulfide (mustard gas, H);  $\beta$ -chloroethyl-benzylsulfide (benzyl-H);  $\beta$ -chloroethyl-ethyl-sulfide (ethyl-H); ethyl-bis( $\beta$ -chloroethyl)amine (EBA); tris( $\beta$ -chloroethyl)-amine (TBA).

### *1. Micro Methods for the Determination of Sulfur and Nitrogen Mustards*

In the present studies, determination of the amount of vesicant vapor penetrated has been made by exposing human skin to the vapor given off by a known amount of liquid vesicant contained in a cup placed on the skin. After a given time interval, the amount of liquid vesicant remaining in the cup is measured, and the amount of vesicant penetrated is determined by difference.

From the work of Henriques, Moritz *et al.* (1) on the penetration of labelled lewisite and mustard gas, it is evident that the rate of penetration of vesicants per cm.<sup>2</sup> of skin surface is of a very low order of magnitude, measurable in terms

TABLE I  
*The Determination of H, Benzyl-H, Ethyl-H, EBA, and TBA*

Compound	Amount taken	Amount found
	$\gamma$	$\gamma$
H.....	677.2	680 $\pm$ 2
Benzyl-H.....	252.7	253 $\pm$ 1
Ethyl-H.....	502.7	498.5 $\pm$ 0.4
EBA-HCl .....	496.2	500 $\pm$ 3
TBA-HCl.....	497.6	504 $\pm$ 2

of gammas. Since the amounts which penetrate are generally small in comparison to the amounts applied to the skin, micro methods of considerable precision were required for our projected study. In order to expedite the work as much as possible, general methods were sought which could be applied to each vesicant with little or no modification. To meet these requirements for measuring the penetration of vesicant vapors, a method has been devised in which the vesicants are hydrolyzed and the liberated H<sup>+</sup> is determined by titration. By this means, amounts of H, benzyl-H, ethyl-H, and the nitrogen mustards (employed as hydrochlorides) in the range of 500  $\gamma$  were determined with an accuracy of 1 per cent. The results are presented in Table I.

In the case of the sulfur-containing mustards, hydrolysis was performed in 50 per cent aqueous alcohol. With the nitrogen mustards, aqueous alkali was employed. In measuring the HCl liberated in the alkaline hydrolysis of the nitrogen mustards by back titration to pH 3-4, the titers obtained represent the difference between the equivalents of HCl liberated and the equivalents of the ethyldiethanolamine or triethanolamine present in the hydrolysate. Supplementary experiments showed that these last named amines can be quanti-

tatively measured by titration to pH 3 (brom phenol blue) with sulfuric acid. The determinations were also successfully carried out on a macro scale.

The accuracy of the results presented above, though not unusually high, is sufficient for the purposes of this investigation. Since, as was mentioned earlier, the penetration rates are determined by difference, the precision of the method as a whole, rather than the absolute accuracy of the titrations is the important factor. As will be shown later, the precision of the method is better than 1 per cent.

*H.*.—A five times recrystallized sample of H made by the thiodiglycol process was kindly put at our disposal by Dr. du Vigneaud.

*Benzyl-H and Ethyl-H.*.—These were stock samples which were freshly distilled before use.

*EBA and TBA.*.—These substances were employed as their hydrochlorides which were dried at room temperature to constant weight *in vacuo* over  $P_2O_5$ . Analyses of these compounds, given below, show that they were pure.

EBA·HCl	$C_6H_{14}NCl_3$	Calculated.	N 6.8, Cl- 17.2
	206.7	Found.	" 6.8, " 17.2, 17.5
TBA·HCl	$C_6H_{14}NCl_4$	Calculated.	N 5.8, Cl- 14.7
	241.2	Found.	" 5.85, " 14.7

For experiments in which the nitrogen bases were employed, these bases were made from the respective hydrochlorides, distilled *in vacuo*, and stored in dry ice until use.

*Ethyldiethanolamine and Triethanolamine.*.—These compounds were commercial preparations. The ethyldiethanolamine was distilled before use.

*Micro Determination of the Sulfur Mustards.*.—One cc. of a standard H, benzyl-H, or ethyl-H solution in alcohol was pipetted into a small flask; an equal volume of water was added, the flask was immediately stoppered and immersed in a water bath at 80° C. for 20 minutes. After cooling to room temperature, a small drop (0.01 cc.) of methyl red was added and the H<sup>+</sup> liberated by hydrolysis was titrated with 0.02 N NaOH by means of a micro burette. A blank was run simultaneously.

*Micro Determination of the Nitrogen Mustard Hydrochlorides.*.—One cc. of the standard aqueous hydrochloride solution was pipetted into a flask, followed by exactly 1 cc. of 0.02 N NaOH. This flask was immediately stoppered and immersed in a water bath at 80° C. for 15 minutes. After cooling to room temperature, 2 drops (*ca.* 0.05 cc.) of brom phenol blue (0.04 per cent in 95 per cent ethanol) were added and the solution was back titrated to a color change (pH 3) by means of 0.01 N HCl. It was later found that methyl red was a more satisfactory indicator than brom phenol blue.

## 2. Apparatus for the Measurement of the Rate of Penetration of Vapors into Skin

*Description of the Apparatus.*.—In preparation for experiments on the penetration of the vapors of vesicants into human skin, an apparatus was designed and constructed for the application of vapors to unit areas of skin. The apparatus consists of the following parts (numbered as in Fig. 1):

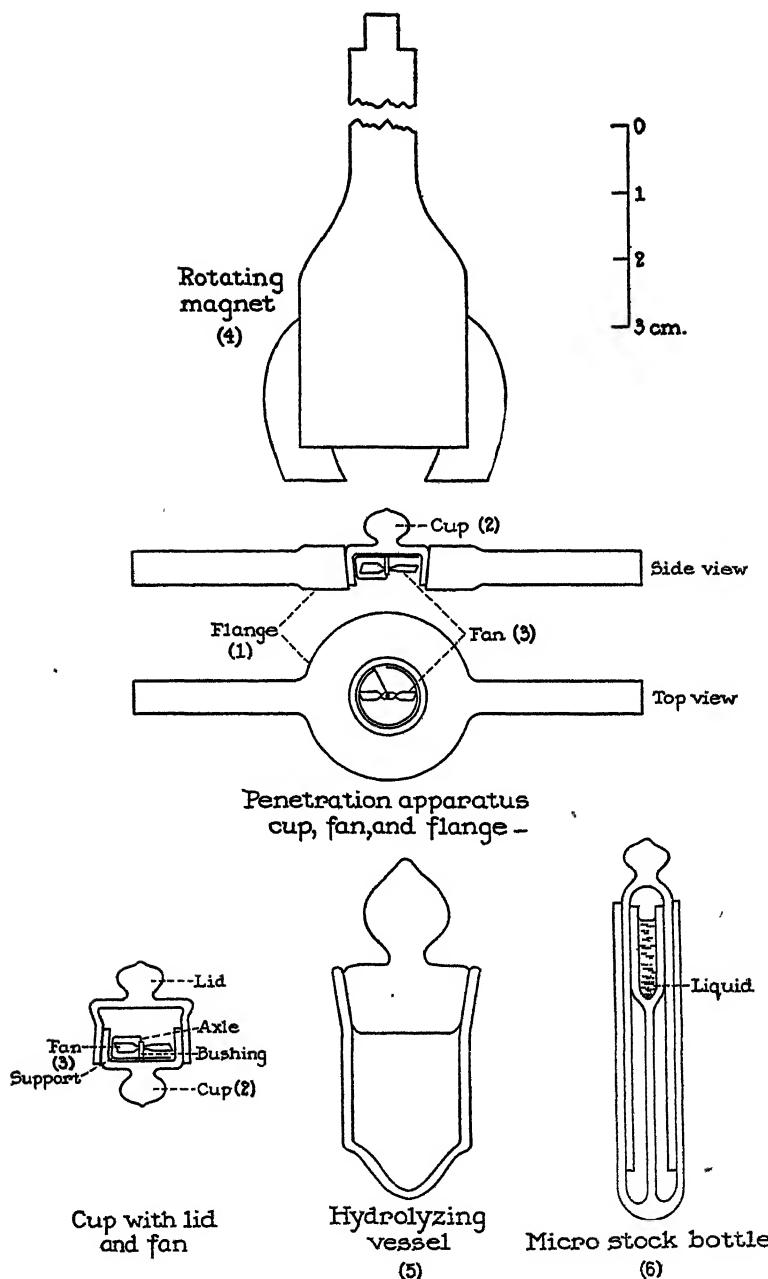


FIG. 1.

(1) A flange, of pyrex glass, measuring 25 mm. in diameter, 5 mm. in thickness, with a hole in the center 18 mm. in diameter at its narrower opening and ground to form a female joint. The top and bottom surfaces are ground flat, and fire-polished. The two arms, 25 mm. long and 4 mm. in diameter, provide a means of attachment to the skin.

(2) A "penetration cup" also of pyrex glass, with thin walls, a small "penny" handle, flat bottom, and a depth of 4 mm. It fits into the flange to fall 1 mm. short of the top surface, and is equipped with a separate ground glass cap.

(3) A fan made of a short length of iron wire fabricated into the conventional propeller design, approximately  $8 \times 2$  mm., with a platinum bushing as its hub. It is necessary that the fan be protected from the action of HCl since this acid is liberated during the hydrolysis procedure. The fan can be rendered acid-proof by electrodeposition of a heavy silver plate followed by a gold plate. A length of platinum wire is suitably shaped to function as the fan axle and support. The wire is looped to a diameter slightly larger than that of the inner base of the cup, is bent vertically up, then horizontally across to the cup center, then down to its bottom, this last portion being the axle proper. A slight groove ground around the base of the inner wall of the cup into which the loop is tightly sprung provides rigid support for the axle.

(4) A small Alnico horseshoe magnet of 30 mm. width, forced into a wooden fork the shank of which is turned down to fit the chuck of a motor. Rotation of the magnet spins the fan.

(5) A hydrolyzing vessel with a long-taper ground joint, and a bottom molded to accommodate the handle of the cup.

(6) A micro stock bottle, made from three selected lengths of tubing that telescope snugly. The innermost sleeve is made into a short handled weighing stick and the stick end then sealed into the rounded off bottom of the outermost sleeve. The middle sleeve is rounded off at one end, provided with a penny handle, and then cut to fit just to the top of the weighing stick and to extend to the bottom of the outer sleeve.

The capillary pipettes (*cf.* Fig. 2 *a*) employed for introduction of the vesicant into the penetration cups are prepared from soft glass, previously cleaned with chromic-sulfuric acid, which is drawn to a bore of 130 to 150 microns. Portions 120 to 150 mm. in length are calibrated roughly with water and are then cut into lengths (30 to 40 mm.) appropriate for delivering 0.5 mg. of vesicant. These sections are sealed into the tapered end of the capillary jacket with Kronig cement, allowing the capillary to protrude about 10 mm.

For ease in handling, and to prevent breakage, sleeve guards are provided for both the capillary pipette and the penetration cups. The sleeve for the pipette is adjusted so that the capillary tip juts slightly beyond its edge. It is also of a diameter that just fits over the container in the micro stock bottle, and therefore acts as a guide in filling. The sleeve for the penetration cup fits over the pipette sleeve at one end, and at the other accommodates the cup. The latter is held firmly by means of a cork grooved to fit the penny handle of the cup (*cf.* Fig. 2 *a, b, c*).

*Description of the Procedure.*—In brief outline, the procedure is as follows: The penetration cup is cleaned and dried, the vesicant is added, and the cup is transferred to the flange previously attached to the skin. After the desired time interval, the

cup is removed from the flange, dropped into the hydrolyzing vessel, and the HCl liberated after hydrolysis is determined. A detailed description follows:

*Cleaning and Handling.*—The cup is cleaned with chromic-sulfuric acid, thoroughly washed with water, and dried. The fan is washed with acetone and dried. The cup, fan, and fan support are handled with forceps.

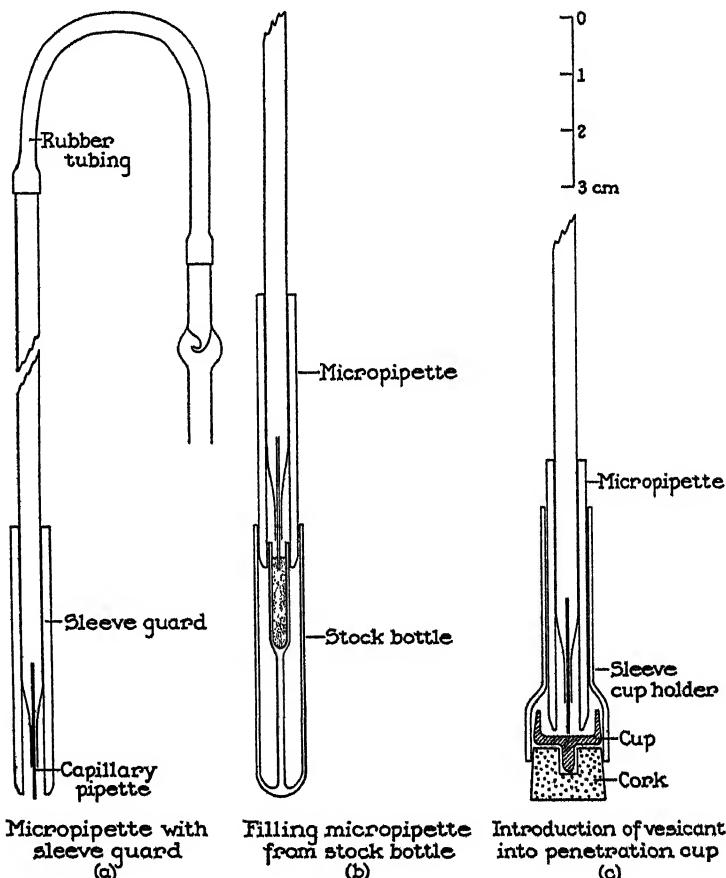


FIG. 2. Apparatus for introduction of vesicant into penetration cup.

*Introduction of Vesicant into Penetration Cup and Subsequent Steps.*—The steps in the addition of the vesicant to the cup are illustrated in Fig. 2 *a* to *c*. The micro pipette is filled by slipping its sleeve guard into the outer sleeve of the stock bottle until the capillary tip touches the liquid vesicant. The pipette fills instantly by capillarity. The vesicant is then pipetted onto a disk of filter paper fitting snugly to the base of the penetration cup; the capillarity of the filter paper is utilized to drain the pipette. This in most cases is a spontaneous process initiated by touching the capillary tip to the filter paper. Occasionally, a slight positive pressure is necessary.

This may be achieved by blowing lightly into a short length of rubber tubing attached to the capillary jacket.

After the addition of vesicant, the cup is immediately transferred to the flange, previously taped to the skin. When the fan is used, the magnet is fixed into position and allowed to rotate at about 600 r.p.m. throughout the course of the experiment. After the chosen time interval, the cup is removed from the flange and dropped gently into the hydrolyzing vessel to which a measured amount (2 cc.) of the hydrolyzing liquid has already been added. The vessel is tightly stoppered and kept in a water bath at 80° C. for 15 minutes, after which it is removed, allowed to cool, and the HCl present after hydrolysis is determined as described earlier.

To determine the reproducibility of delivery from the micro pipettes, they are filled with the vesicant, as described above, and then emptied directly into the hydrolyzing vessel. The HCl liberated upon hydrolysis is determined as described before. It is found that, with some practice, it is possible to deliver 500 γ of H to within about  $\pm 1\gamma$ . With the vesicants other than H, the precision is somewhat less, namely,  $\pm 1$  to  $2\gamma$ .

*Control Experiments.*—To determine the incidental losses of vesicant vapor during the penetration experiments, parallel controls were always run. In these control experiments, the manipulations were the same as in the penetration experiments except that the penetration cup was closed off for the requisite period by means of a ground glass cap. In the case of H, the loss in the control experiments was found to be 7 γ in 10 minutes, 10 γ in 20 minutes, and 12 γ in 30 minutes. Similar data were accumulated for the other vesicants studied.

It has been found that the losses in the control experiments vary not only with the duration of the experiment, but also with the temperature at which the experiment is conducted. Moreover, the magnitude of the loss is different for each agent, varying as a function of volatility. Therefore, in the penetration experiments with human subjects, reported later, the amount of vesicant penetrated represents the difference between the amount of agent left in the cup after a given exposure period and a control value. The control value, or blank, is the average amount of agent left in the cup as determined in a series of control experiments. To obtain each blank value with a high degree of precision, a set of at least 12, and sometimes as many as 40, control experiments were performed. To illustrate the type of data obtained, a few typical examples may be given. In the penetration experiments with H vapor given in Table IV under "20 minute exposure," the blank was 514 γ. This blank was the mean of 30 control experiments whose average deviation (A.D.) was  $\pm 1.5\gamma$ . The standard deviation (S.D.)<sup>1</sup> in this series of experiments was  $\pm 1.8\gamma$ , and the standard error (S.E.)<sup>1</sup> of the mean was  $\pm 0.33\gamma$ . Similarly, the "10 minute blank" employed in the experiments with EBA vapor given in Table V under "10 minute exposure" was 514γ. This blank was the mean of 18 controls having an A.D. of  $\pm 2$ , an S.D. of  $\pm 2.8\gamma$ , and a S.E. of  $\pm 0.7\gamma$ . The "60 minute blank" employed in the experiments

<sup>1</sup>The standard deviation (S.D.) was computed according to the equation S.D. =  $\sqrt{\sum d^2/(n-1)}$  and the standard error (S.E.) was computed according to the equation S.E. =  $\sqrt{2d^2/[n(n-1)]}$  where  $d$  represents the deviation of each value from the mean, and  $n$  represents the number of values.

with TBA vapor given in Table VI under "60 minute exposure" was  $510\gamma$ . This blank was the mean of 18 controls having an A.D. of  $\pm 1.6\gamma$ , an S.D. of  $\pm 1.7\gamma$ , and an S.E. of  $\pm 0.4\gamma$ .

*Subsaturation Vapor Concentrations.*—A procedure has been devised for the establishment within the penetration cup of known subsaturation vapor concentrations of vesicants. This method is based on the finding that solutions of H (or ethyl-H) in *n*-dibutylphthalate obey Raoult's law. As shown by de Heen (2) and Hine (3), the rate of evaporation of a liquid from a solution is proportional to the vapor pressure of the liquid in the solution. Since the vapor pressures of the pure vesicants are known, a comparison of the rate of evaporation of a given vesicant-dibutylphthalate mixture with that of the pure vesicant in question is a measure of the vapor pressure of the vesicant in the mixture. Measurement of the rate of evaporation of H (or ethyl-H) has shown that for mol fractions of 0.10 to 1.00, the rate is directly proportional to the mol fraction of the vesicant in dibutylphthalate solution.

### 3. Model Experiments on the Rate of Absorption of H Vapor

In the previous section, a description was given of the design and operation of an apparatus for measuring the penetration of vesicants and other vapors into skin. In order to determine the limitations and sources of error which are involved in the use of this apparatus, model experiments have been performed in which non-volatile solvents replaced skin as the absorbent surface.

In each experiment performed at constant temperature and humidity, a measured amount of H (usually  $600\gamma$ ) was pipetted into the penetration cup described previously. Immediately afterwards, the cup was inserted into its ground glass lid, the inside bottom of which contained 0.05 cc. of the absorbent. After the desired time interval, the lid was removed, dropped into a hydrolyzing vessel, water or 50 per cent acetone was added, and the contents of the vessel were heated at  $80^\circ\text{C}$ . for 15 minutes. The H absorbed by the solvent was determined by titrating the HCl liberated during the hydrolysis. In some instances, supplementary analyses were made to measure the H remaining in the penetration cup. The absorption values thus obtained by difference agreed closely (within a few gamma) with those obtained by direct analysis of the solvents.

It was found that the rate of evaporation of liquid H was markedly increased when the surface of H was increased by spreading it on filter paper. It is evident from the data in columns 1 and 2 of Table II that the increase in the surface of liquid H markedly affects the rate of its evaporation into diethylene glycol. As will be noted from columns 1 and 2 of Table III, a similar result was obtained when vaseline was used as the absorbent.

In order to determine the effect of change in the rate of transfer of H vapor from the liquid H to the absorbing surface of diethylene glycol, the rate of absorption into diethylene glycol was determined both when the H vapor was allowed to diffuse by itself and when the H vapor was agitated by fanning. In control experiments it was found that when the fans were operated for periods

TABLE II  
*Absorption of H Vapor by Diethylene Glycol*

H in penetration cup,  $596 \pm 2 \gamma$ .  
 Temperature,  $22^\circ\text{C}.$ ; relative humidity, 32 per cent

Time	Amount of H absorbed by diethylene glycol					
	Evaporation from H droplet		Evaporation from H on filter paper			
	Diffusion (1)		Diffusion (2)		Fanning (3)	
	Total	Average per min.	Total	Average per min.	Total	Average per min.
min.	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$
15			47	3.2	274	18.3
			50			
30	39	1.3	88	3.2	412	13.7
	41		104			
60	69	1.15	175	3.15		
	69		203			
120			277	3.2		
			386			
150	141	1.0				
	156					

TABLE III  
*Absorption of H Vapor by Vaseline*

H in penetration cup,  $596 \pm 2 \gamma$ .  
 Temperature,  $22^\circ\text{C}.$ ; relative humidity, 32 per cent.

Time	Amount of H absorbed by vaseline					
	Evaporation from H droplet		Evaporation from H on filter paper			
	Vaseline film		Vaseline film			Vaseline on filter paper
	Diffusion (1)		Diffusion (2)	Fanning (3)		Diffusion (4)
	Total	Average per min.	Total	Average per min.	Total	Average per min.
min.	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$
15			13	0.9	37	2.5
30	21	0.7	38	1.3	35	1.2
45			46	1.0	39	0.9
60	27	0.45				

TABLE IV

*Rate of Penetration of H Vapor into Human Skin*

Temperature, 21-23 C., relative humidity, 44 to 46 per cent; area of skin exposed, 1.3 cm.<sup>2</sup> of flexor aspect of the left or right forearm; acclimatization time, 30 minutes. The lesions were evaluated 48 hours after exposure unless otherwise indicated.

Subject	Site	3 min. exposure		6 min. exposure		10 min. exposure	
		Amount penetrated	Lesion	Amount penetrated	Lesion	Amount penetrated	Lesion
E. E. M.	L	γ	E	γ		16	V
	R	6	E	12	V		
T. M. C.	L	6	E	13	V	22	VR
	R	4	E	14	V		
H. A. T.	L	4	E	14	V	20	VR
	R	4	E	16	V		
M. L.	L	3	E	16	V		
	R	—	E	17	V		
W. D. M.	L	4	E-	12	E		
	R	4	E-	14	E		
H. H. H.	L	—	E	10	V		
	R	6	E-	13	V		
B. B. W.	L					19	V
	R					14	V
A. L. G.	L					18	V
	R					22	V
W. W. M.	R					17	V, N
	R					19	V
J. F. M.	R					22	V
	R					22	V
Average .....		4		13		19	
Average deviation .....		±1		±1.5		±2.2	
Standard deviation .....		±1.4		±2.2		±2.8	
Standard error .....		±0.4		±0.7		±0.8	
Penetration rate:							
Cm. <sup>2</sup> per min. ....		1.0		1.67		1.46	
Standard error .....		±0.1		±0.09		±0.06	

O, no reaction. PV, pinhead vesicles. N, necrosis.

E-, mild erythema. V, vesication.

E, erythema. VR, vesicular rim.

up to 1 hour in the absence of any absorbent, the initial amount of H was recovered quantitatively from the penetration cup.

It will be noted from the data in columns 2 and 3 of Table II that the rate of absorption was markedly increased when the H vapor was agitated. Apparently the absorbing surface is not in contact with a saturated H vapor when the fanning is omitted. The influence of the rate of fanning of H vapor on its

TABLE IV—Concluded

Subject	Site	20 min. exposure		30 min. exposure	
		Amount penetrated	Lesion	Amount penetrated	Lesion
A. W. H.	L	γ		γ	
	R	33	NVR		
M. H.*	L	35	NVR		
	R	33	N		
E. E. W.	L	37	N		
	R	25	NVR		
J. F. W.	L	30	NVR		
	R	46	NVR		
E. S. W.	L	40	NVR		
	R	36	NVR		
E. P. A.	L	38	V		
	R	38	NVR		
L. J. B.	L‡	38	NVR		
	R	34	NVR		
J. E. F.	L‡	42	V		
	R	43	N		
H. S.*	L‡	33	V		
	R	35	V		
S. A. D.	L‡	33	NVR		
	R	40	NVR		
B. B. W.	L‡	39	NVR		
	R	33	V		
A. L. G.	L‡	31	NVR		
	R	35	NVR		
W. W. M.	L	33	V	46	NVR
				53	NVR
J. F. M.	L			55	NVR
E. G. R.	L			61	NVR
	R				
Average.....		36		54	
Average deviation.....		±3.5		±4	
Standard deviation.....		±4.5		±6	
Standard error.....		±0.9		±3	
Penetration rate:					
Cm. <sup>2</sup> per min.....		1.38		1.38	
Standard error.....		±0.04		±0.08	
Mean penetration rate for H vapor: 1.4 γ/cm. <sup>2</sup> /min.; standard error ±0.06 γ					

\* These subjects were Negroes.

‡ In these experiments, the H vapor was fanned.

absorption by an absorbent was also studied. It was found that the rate of absorption of H is increased from 6.2 γ/minute at 300 R.P.M. to 13.4 γ/minute at 900 R.P.M.

It was to be expected that the absorption of H by an absorbent would be a function of the surface area of the absorbent. In order to test this hypothesis, vaseline was employed as the absorbent; in one determination a film of vaseline was deposited by melting vaseline on a glass surface, while in a second determination, the vaseline was spread on filter paper, thus increasing its surface. It will be noted from columns 2 and 4 of Table III that the absorption of H vapor into vaseline was markedly influenced by the surface area of the absorbent.

An indication of the influence, on the rate of absorption, of the rate of diffusion of H from the surface of the absorbent into the body of the absorbent emerges from the data in column 3 of Table III. It will be noted that when the H vapor was agitated and allowed to be absorbed by the vaseline film, the absorption was practically complete within 15 minutes. It may be concluded that the surface of the vaseline film has become saturated with H within 15 minutes and that the diffusion from the surface layer of vaseline into the body of the vaseline layer is very slow. In order to check this conclusion, similar experiments were performed in which the depth of the vaseline film was varied. It was found that there was no significant difference in the amounts of H absorbed when the thickness of the vaseline film was varied tenfold.

#### *4. Penetration of Vesicant Vapors into Human Skin*

The experimental studies recorded in this section were performed on a total of one hundred human volunteers during the period, May to November, 1944.

The exposures to vesicant vapors were made in an air-conditioned room maintained at constant temperature and humidity. For the experiments at 21-23°C., the subjects were kept at the temperature of the room for 30 to 45 minutes before exposure to the vesicant vapors; for the experiments at 30-31°C., the preliminary period was 1 hour. The flanges were then taped to the flexor aspect of both forearms of each subject. Immediately after the introduction of the vesicant into the penetration cup, the cup was inserted into the flange and allowed to remain there for the desired time interval. In some experiments the vapor was agitated by means of the magnetically driven fan described earlier. The cup then was removed and dropped into the hydrolyzing liquid. The amount of vesicant remaining in the cup was determined. The amount of agent which had penetrated was taken as the difference between the control value and the amount of agent left in the cup after exposure of the skin to the vapor.

*The Rate of Penetration of H, EBA, TBA, Benzyl-H, and Ethyl-H into Human Skin at 21-23°C.*—The results obtained when the subjects were exposed to the action of H vapor for periods ranging from 3 to 30 minutes are given in Table IV. It will be noted from Fig. 3 that there is a linear relationship between the amount penetrated and the time of exposure. The mean rate of penetration, under the conditions of the experiment, is about 1.4 γ per cm.<sup>2</sup> per

minute. This figure has a standard error of  $\pm 0.06\gamma$ , which is about 4 per cent of the rate. It should be mentioned that there is little difference in the two values obtained on symmetrical sites for each individual and likewise that there is little variation when the values from subject to subject are compared. It is also of some interest to note that no significantly different results were obtained with the two subjects who were Negroes.

The effect of agitation of the H vapor was studied in a group of experiments in which six subjects were employed. The results, presented in Table IV, show that the rate of penetration into human skin is not influenced by agitation of the H vapor. This indicates that the rate of transfer of H vapor from the

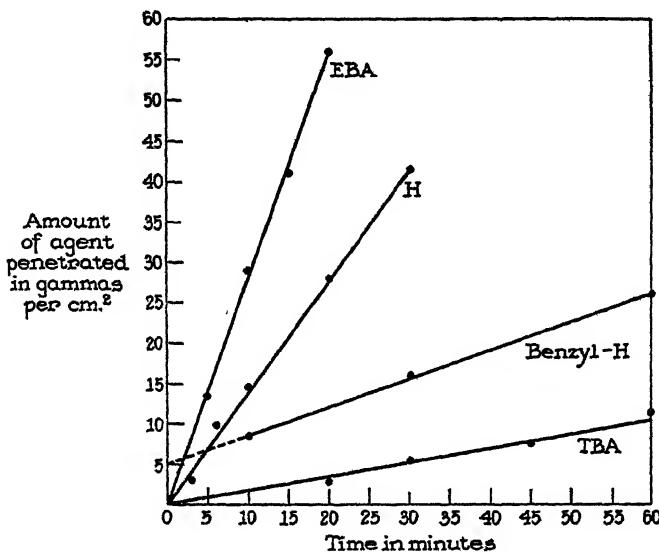


FIG. 3. Penetration of vesicant vapors into human skin at 21-23°C.

liquid H present in the bottom of the cup to the surface of the skin is more rapid, even without fanning, than is the rate of absorption of the vapor by the skin.

The results of the penetration measurements of EBA vapor at a temperature of 22°C. and a relative humidity of 50 to 52 per cent are presented in Table V. It will be noted from the table and from Fig. 3 that the mean rate of penetration of EBA vapor under these conditions is  $2.8\gamma/\text{cm}^2\text{ per minute}$  (standard error,  $\pm 0.07\gamma$ ) and that the amount penetrated is linear with time.

The effect of fanning the EBA vapor for 5 and 10 minute periods was also studied. As shown in Table V, agitation of the EBA vapor had no influence on the rate of penetration. This would indicate that the rate of transfer of the EBA vapor from the liquid in the bottom of the penetration cup to the surface of the skin is not a limiting factor in the measurement of the penetration rate.

TABLE V

*Rate of Penetration of EBA Vapor into Human Skin*

Temperature, 22°C.; relative humidity, 50 to 52 per cent. Acclimatization time, 45 minutes. The sites exposed and the symbols used to describe the lesions 48 hours after exposure are the same as those given in Table IV.

Subject	Site	5 min. exposure		10 min. exposure		15 min. exposure		20 min. exposure	
		Amount penet- rated	Lesion	Amount penet- rated	Lesion	Amount penet- rated	Lesion	Amount penet- rated	Lesion
J. M.	L	15	O	36	E-	γ		γ	
	R	17	O	32	E				
W. J. H.	L	20	E-	42	E-				
	R	15	E-?	38	E				
B. B. W.	L	17	PV	36	V				
	R	20	PV	39	V				
H. H. W.	L	15	E			60	PV		
	R	19*	E-			56	PV		
J. M. T.	L	17*	E-			51	PV		
	R	17	E-			47	PV		
C. G. J.	L	21*	E-			51	V		
	R	21	E-			51	V		
J. H. M.	L			40	PV			77	V
	R			40*	PV			77	V
H. A. W.	L			37	PV			70	V
	R			38*	PV			72	PV
F. S.	L			37	V			74	V
	R			41*	V			74	V
H. F. M.	L					48	PV	77	PV
	R					51	PV	64	V
H. B. G.	L					53	E	70	E-
	R					55	PV	73	PV
F. M. G.	L					55	PV		
	R					55	PV		
Average.....		17.5		38		53		73	
Average deviation.....		±2		±2		±3		±3	
Standard deviation.....		±2.3		±2.5		±3.7		±4	
Standard error.....		±0.7		±0.5		±1.1		±1.3	
Penetration rate:									
Cm. <sup>2</sup> per min.....		2.8		2.9		2.7		2.8	
Standard error.....		±0.1		±0.05		±0.06		±0.05	

Mean penetration rate for EBA vapor:  $2.8\gamma/\text{cm}^2/\text{min.}$ ; standard error  $\pm 0.07\gamma$

\* In these experiments the EBA vapor was fanned.

The results of the penetration measurements of TBA at a temperature of 22–23°C. and a relative humidity of 45 to 48 per cent are presented in Table VI.

TABLE VI  
*Rate of Penetration of TBA Vapor into Human Skin*

Temperature, 22–23°C.; relative humidity, 45 to 48 per cent. Acclimatization time, 30 minutes. The sites exposed and the symbols used to describe the lesions 48 hours after exposure are the same as those given in Table IV.

Subject	Site	20 min. exposure		30 min. exposure		45 min. exposure		60 min. exposure	
		Amount penet- rated	Lesion	Amount penet- rated	Lesion	Amount penet- rated	Lesion	Amount penet- rated	Lesion
W. E. V.	L	γ		γ	E-, E	γ		γ	
	R	3	E-?	8, 4		8	V		
J. S. P.	L			5, 5	E-, E				
	R	3	E-			11	E		
E. A. S.	L			8, 4	E, E-				
	R	7	E			8	V		
H. L. D.	L			7	V				
J. F. M.	L			9	E				
	R			7	E				
W. G. G.	L			9	E + 1PV				
	R			10	E				
W. A. D.	L					—	V	16	V
	R					12	V	—	V
F. J. R.	L					10	V	12	VR
	R					14	V	14	V
H. W. S.	L					10	E	—	V
	R					10	V	17	VR
N. W. T.	L							18	V
	R							17	V
C. K. N.	L							11	E
	R							11	E
J. R. M.	L							18	V
	R							17	V
Average.....		4		7		10		15	
Average deviation.....		—		±1.7		±1		±2.4	
Standard deviation.....		—		±2		±1.7		±2.9	
Standard error.....		—		±0.6		±0.5		±0.9	
<hr/>									
Penetration rate:									
Cm. <sup>2</sup> per min.....		0.15		0.18		0.17		0.19	
Standard error.....		—		±0.02		±0.01		±0.01	

Mean penetration rate for TBA vapor:  $0.18 \gamma/\text{cm}^2/\text{min.}$ ; standard error  $\pm 0.01 \gamma$   
(20 min. exposure not included)

It will be noted from the table and from Fig. 3 that the mean rate of penetration of this vesicant is  $0.18 \gamma$  per  $\text{cm}^2$  per minute (standard error,  $\pm 0.01 \gamma$ ) and, as

in the case of H, the amount penetrated is linear with time. Measurements were also attempted after a 10 minute exposure period, but here the amount penetrated was too small to be measured (less than 5  $\gamma$ ) and the clinical observations (see Table VI) showed either a very mild erythema or none at all.

In view of the length of the exposure time necessary to get a measurable amount of TBA vapor to penetrate the skin, agitation of the TBA vapor inside the cup was impracticable. As will be shown later, fanning of benzyl-H vapor does not alter the penetration rate of this agent. Since benzyl-H has about the same volatility as HN<sub>3</sub>, and has, in addition, a higher penetration rate than TBA, there is every reason to believe that the rate of transfer of TBA vapor from the surface of the liquid in the bottom of the penetration cup to the surface of the skin is not a limiting factor in these experiments.

The results obtained when subjects were exposed to the vapor of benzyl-H at a temperature of 22°C. and a relative humidity of 55 to 60 per cent for periods ranging from 10 minutes to 1 hour are given in Table VII and are plotted in Fig. 3. It will be noted that while the amount penetrated is linear with time between the exposure times of 10 and 60 minutes, the slope of the line is such that it intersects the ordinate at 5  $\gamma$  and not at the origin. It has been found that this deviation is not due to the presence in our sample of benzyl-H of a volatile chlorine-containing impurity which evaporates off rapidly. This possibility was excluded in a control experiment in which the free diffusion (in air) of our sample of benzyl-H was followed. The rate of evaporation was strictly linear and the plot passed through the origin. In order to calculate the rate of penetration of benzyl-H, it is necessary, therefore, to calculate the slope of the plot in Fig. 3. In this manner it is found that the rate of penetration of benzyl-H is about 0.35  $\gamma$  per cm.<sup>2</sup> per minute.

It appears probable that the anomaly observed in the penetration data on benzyl-H is due to a rapid adsorption of an appreciable quantity of benzyl-H on the surface layer of the skin, and the retention of this quantity of benzyl-H on the skin surface during the exposure to the vapor. This adsorption appears to be so rapid that it is completed during the initial 10 minutes of the exposure period.

As may be noted from Table VII, agitation of the benzyl-H vapor did not influence the rate of penetration significantly. This would indicate, as in the case of H, that the rate of transfer of benzyl-H vapor from the liquid in the bottom of the penetration cup to the surface of the skin is not a limiting factor in these measurements.

The rate of penetration of ethyl-H was also studied. In view of the high volatility of this agent (about 22 times that of H), it was necessary to perform each operation of the experimental procedure in as reproducible a manner as possible. When each operation was carefully timed, it was found that successive controls differed by only a narrow margin (less than 10  $\gamma$ ). The results

obtained with human volunteers are given in Table VIII. It will be noted that the individual values for the 2.5 minute exposures show the agreement to be expected from the experimental procedure used, but there is a great variation in the individual values for the 5 and 10 minute exposures. This cannot as yet

TABLE VII  
*Rate of Penetration of Benzyl-H Vapor into Human Skin*

Temperature, 22°C.; relative humidity, 55 to 60 per cent. Acclimatization time, 45 minutes. The sites exposed and the symbols used to describe the lesions 48 hours after exposure are the same as those given in Table IV.

Subject	Site	10 min. exposure		30 min. exposure		60 min. exposure	
		Amount penetrated	Lesion	Amount penetrated	Lesion	Amount penetrated	Lesion
S. B. I.	L	γ	0	γ		γ	
	R	11	0				
M. F. G.	L	15	0				
	R	19	0				
D. R. M.	L	15	0				
	R	15	0				
R. E. P.	L			22	E-		
	L			18	E-		
	R			22	E-		
J. C. O.	L			22	E-		
	R			22	E-		
	R			18	E-		
R. L. U.	L	7	0			33	E
	R	11*	0			33	E
F. A. F.	L	7*	0			35	E-
	R	7	0			35	E
M. P. E.	L	7*	0			33	E-
	R	11	0			33	E-
Average.....		11		21		34	
Average deviation.....		±1.7		±1.7		±1.7	
Standard deviation.....		±2.1		±2.1		±2.1	
Standard error.....		±0.9		±0.9		±0.9	

\* In these experiments the benzyl-H vapor was fanned.

be satisfactorily explained, but it does not seem to be due to experimental error, since each step in the experimental technique was carefully checked for accidental loss of ethyl-H vapor, but none was found which would account for the variation in penetration values.

It is of considerable interest that the mean values of the loss of ethyl-H from the penetration cup should be essentially the same after exposure times of 2.5

## PENETRATION OF VESICANT VAPORS

5 minutes. As will be noted from Table VIII, however, the lesions produced after 2.5 minutes were much less severe than those produced after 5

TABLE VIII

*Rate of Penetration of Ethyl-H Vapor into Human Skin*

Temperature, 22°C.; relative humidity, 46 to 55 per cent. Acclimatization time, 45 min.

The sites exposed and the symbols used to describe the lesions 48 hours after exposure are the same as those given in Table IV.

Subject	Site	2.5 min. exposure		5 min. exposure		10 min. exposure*	
		Amount penetrated	Lesion	Amount penetrated	Lesion	Amount penetrated	Lesion
C. W. M.	R	70	E	82	V	110	V
	L	70	E				
R. A. G.	R	70	E	56	V	119	V
	L	67	E				
G. T. R.	R	67	E	94	V	165	V
	L	48	E-				
H. G. F.	R	51	E-	72	V	53	V
	L	67	E				
W. E. S.	R	51	E-	74	V	60	V
	L	67	E				
E. F. L.	R	51	V	128	V	128	V
	L	59	V				
A. M. B.	L	80	E	53	V	74	V
	R	69	V				
J. C. K.	L	37	V	60	V	98	V
	R	42	V				
Average.....		62		63		98	
Average deviation.....		±8.5		±16		±33	

\* Immediately after a 10 minute exposure to ethyl-H vapor, all the subjects exhibited erythema at the exposure sites. With the other agents studied thus far, there has been no immediately visible reaction of this kind.

minutes, thus indicating that more ethyl-H had penetrated into the skin in 5 minutes than in 2.5 minutes. This raises the question as to whether the skin is capable of rapidly adsorbing on its surface an appreciable quantity of ethyl-H which slowly penetrates into the skin. Removal of the penetration cup

after 2.5 minutes would allow much of the volatile ethyl-H to evaporate before penetration into the skin; after 5 minutes' exposure, however, a larger fraction of the adsorbed ethyl-H would have penetrated into the skin. If this is indeed the case, then the amount of ethyl-H lost from the penetration cup would not be a measure of penetration alone but rather would be a measure of both adsorption on the surface of the skin and penetration into the skin.

It should be mentioned that Smith *et al.* (4) have presented evidence to indicate that, following exposure of skin to saturated H vapor, some of the vesicant evaporated from the skin surface after removal of the penetration cup. There has not been an opportunity to study quantitatively the postexposure evaporation from the skin of any of the vesicant vapors employed in our work. In view of the fact that H, benzyl-H, EBA, and TBA have a much lower volatility than ethyl-H, it seems probable that postexposure evaporation would have a less important effect on the amount penetrated into the skin for these four agents than it would in the case of ethyl-H. This question requires further careful investigation.

*The Rate of Penetration of H, EBA, and TBA Vapors into Human Skin at 30–31°C.*—It is generally agreed that vesicants become more effective with increasing temperature. It becomes of interest to ascertain, therefore, whether this greater effectiveness is due mainly to the increased volatility of the vesicants, or whether an enhanced sensitivity of the skin at the higher temperature also plays a rôle. To study this question, experiments with human subjects were performed at a temperature of 30–31°C. and a relative humidity of 47 to 49 per cent. At this temperature the sweat glands begin to function (5) and the skin is moist. This fact was established by observation of the volunteers, and by preliminary experiments which demonstrated that appreciable amounts of moisture condense inside the penetration cups.

In Tables IX, X, and XI are given the results of the penetration measurements for H, EBA, and TBA vapors at a temperature of 30–31°C. and a relative humidity of 47 to 49 per cent. As will be noted from the tables and from Fig. 4, the amount penetrated for each vesicant was found to be linear with time. The mean penetration rates were: for H vapor,  $2.7 \text{ } \gamma/\text{cm.}^2/\text{minute}$  (standard error,  $\pm 0.11 \text{ } \gamma$ ); for EBA vapor,  $5.1 \text{ } \gamma/\text{cm.}^2/\text{minute}$  (standard error,  $\pm 0.15 \text{ } \gamma$ ); for TBA vapor,  $0.29 \text{ } \gamma/\text{cm.}^2/\text{minute}$  (standard error,  $\pm 0.015 \text{ } \gamma$ ).

The fact that the rate of penetration was found to be independent of time for all three agents indicates that the amount of moisture which condensed in the penetration cup was not sufficient to affect the penetration rate. Had the vesicant been diluted gradually with, or covered by, water, a progressively reduced vapor concentration in the cup should have resulted, and a gradual decrease in the penetration rate would have been observed.

Three of the subjects used in the EBA measurements were Negroes. As will be noted from the data of Table X, the penetration rate for these subjects was

TABLE IX  
*Rate of Penetration of H Vapor into Human Skin*

Temperature, 30.6°C.; relative humidity, 48 to 49 per cent. Acclimatization time, 1 hour. The sites exposed and the symbols used to describe the lesions 48 hours after exposure are the same as those given in Table IV.

Subject	Site	2 min. exposure		4 min. exposure		6 min. exposure	
		Amount penetrated	Lesion	Amount penetrated	Lesion	Amount penetrated	Lesion
R. C. W.	L	γ	E	γ	V	γ	
	R	5	E	16	V		
A. J. P.	L	7	E	13	V		
	R	7	V	14	V		
F. R. A.	L	7	V	15	V		
	R	8	V	15	V		
M. L. S.	L	8	PV	14	V	20	V
	R	7	V				
M. S.	L	6	V	13	V	18	V
	R	9	V				
A. J.	L	5	E + 1PV	13	V	22	V
	R	9	V				
W. O. B.	L	7	E	13	V	25	VR
	R	8	V				
R. W.	L	5	V	14	V	20	VR
	R	7	V				
R. E. B.	L	8	V	16	VR	20	VR
	R	5	V				
W. G.	L	—	E + 1PV	—	V	—	
	R	—	E				
S. D. R.	L	—	E	—	V	—	
	R	—	E				
A. H. R.	L	—	E	—	V	—	
	R	—	PV				
Average .....		7		14		21	
Average deviation.....		±1		±1		±1.8	
Standard deviation.....		±1.4		±1.3		±2.6	
Standard error.....		±0.3		±0.4		±1	
Penetration rate:							
Cm. <sup>2</sup> per min.....		2.7		2.7		2.7	
Standard error.....		±0.13		±0.07		±0.13	

Mean penetration rate for H vapor: 2.7 γ/cm.<sup>2</sup>/min.; standard error ±0.11γ

no different from that of the white subjects. It will be recalled that H vapor at 21–22°C. was found to penetrate the skin of two Negro subjects at the same rate as that of white individuals and to produce the same clinical response in the

TABLE X

*Rate of Penetration of EBA Vapor into Human Skin*

Temperature, 30–31°C.; relative humidity, 47 to 49 per cent. Acclimatization time, 1 hour. The sites exposed and the symbols used to describe the lesions 48 hours after exposure are the same as those given in Table IV.

Subject	Site	2.5 min. exposure		5 min. exposure		7.5 min. exposure		10 min. exposure	
		Amount pene- trated	Lesion	Amount pene- trated	Lesion	Amount pene- trated	Le- sion	Amount pene- trated	Lesion
G. A. S.*	L	γ		γ		γ		γ	
	R	19	O	37	O	52	O	73	E-
W. H. L.*	L	14	O	34	O	56	O	73	O
	R	19	O	27	O	68			E
M. W. V.*	L	16	O	31	O	52	E-	68	E + PV(?)
	R	19	O	37	O	48	E	66	E
C. M. S.	L	15	V	30	V	48	E	62	E-
	R	18	V	28	V	45	V		
B. M. F.	L	15	E-	31	V	51	V		
	R	18	E	28	V	45	V		
C. H.	L	12	O	33	E	51	V		
	R	18	O	31, 34	E-, E-	47	V		
H. L. D.	L	16	E-?	31, 31	V, V	52	V		
	R	12	E	34, 37	V, E-	49	V		
E. H. E.	L	19	E-	37, 33	E, E	53	V		
	R	19	E-	37, 33	V, V	53	V		
D. R. C.	L	16	E-	37, 33	V, V	55	E		
	R	19	E-	33, 33	E-, E	55	E		
F. M. H.	L	16	E-	37, 33	V, V	50		68	
	R	19	E-	33, 33	E-, E	53		68	
L. R. R.	L	16	E-	37, 33	V, V	53		68	
	R	19	O	33, 33	E-, E	55		68	
R. L. C.	L	19	O	33, 33	E-, E	55		68	
	R	19	O	33, 33	E-, E	55		68	
Average.....		17		33		50		68	
Average deviation.....		±2.5		±2.5		±3		±3	
Standard deviation.....		±3.2		±3.3		±3.5		±3.7	
Standard error.....		±0.8		±0.7		±0.9		±1.7	
Penetration rate:									
Cm. <sup>2</sup> per min.....		5.2		5.1		5.1		5.2	
Standard error.....		±0.25		±0.1		±0.1		±0.15	

Mean penetration rate for EBA vapor: 5.15γ/cm.<sup>2</sup>/min.; standard error ±0.15γ

\* These subjects were Negroes.

Negroes and the whites; i.e., vesication and necrosis resulted in both. On the other hand, the clinical response of the three Negroes to EBA vapor was strik-

ingly different from that of the white subjects. Whereas the white subjects blistered in 50 per cent of the sites after a 5 minute exposure (Table X), the

TABLE XI  
*Rate of Penetration of TBA Vapor into Human Skin*

Temperature, 30°C.; relative humidity, 47 to 48 per cent. Acclimatization time, 1 hour. The sites exposed and the symbols used to describe the lesions 48 hours after exposure are the same as those given in Table IV.

Subject	Site	15 min. exposure		30 min. exposure		45 min. exposure	
		Amount penetrated	Lesion	Amount penetrated	Lesion	Amount penetrated	Lesion
J. F. G.	L	7	E	12	V	γ	
	R	5	V	11	VR		
G. E. T.	L	7	PV	12	V		
	R	7	PV	10	V		
J. S.	L	9	V	10	VR		
	R	7	V	10	VR		
H. E. K.	L	5	E-			16	V
	R	7	E			17	V
E. N. F.	L	7	V			18	N
	R	5	V			16	VR
G. F. B.	L	5	E-			17	VR
	R	5	V			19	VR
D. D. B.	L			10, 10	V, V	18	VR
	R	5	E				
E. E. A.	L			12, 10	V, V	16	V
	R	5	E				
E. A. L.	L			12, 10	V, V	18	V
	R	7	E				
Average.....		6		11		17	
Average deviation.....		±1		±1		±1	
Standard deviation.....		±1.3		±1.2		±1.2	
Standard error.....		±0.4		±0.3		±0.4	
<hr/>							
Penetration rate:							
Cm. <sup>2</sup> per min.....		0.31		0.28		0.29	
Standard error.....		±0.2		±0.01		±0.01	

Mean penetration rate for TBA vapor: 0.295 γ/cm.<sup>2</sup>/min.; standard error ±0.015γ

Negro subjects failed to show vesication after an exposure period of 10 minutes. The previous failure to observe any difference in clinical response in the case of H vapor may have been due to the fact that the exposure period used for the Negro subjects was more than four times that required to produce 50 per cent vesication in the white subjects. For this reason, differences between the two groups, less than three- or fourfold in extent, would not have been detected.

Casual inspection of the data given in Tables IX to XI reveals a surprising lack of individual variations in the amounts of a given agent penetrated in a given time and at a given temperature. In other words, biological variation appears to play a negligible rôle in the penetration of vesicant vapors into human skin. This impression is strongly reinforced by the statistical data appended to each table. Thus the average deviation, standard deviation, and standard error are scarcely larger for any set of penetration values than they are for the typical controls given earlier. The small variations observed border on the limits of experimental error, and cannot be attributed to biological

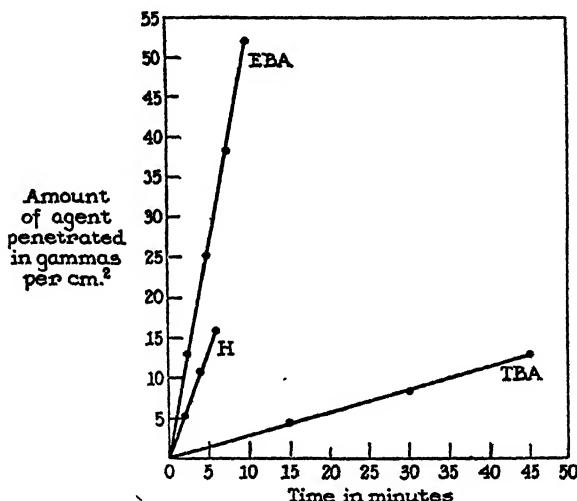


FIG. 4. Penetration of vesicant vapors into human skin at 30-31°C.

differences. The standard error of the mean penetration rate is, in all cases, small, amounting to 10 per cent or less of the value.

The individual variation in clinical response to a given dose of vesicant is much more difficult to determine with certainty, since the clinical response can only be evaluated qualitatively, or at best, semiquantitatively. Such obvious phenomena as the resistance of Negro skin to damage by EBA vapor, and the dramatic response of hypersensitive individuals were noted in the course of the work.

##### *5. Correlation of Penetration Rates and Volatilities*

It will be seen from Table XII that, in the case of H, the increase in penetration rate from 21-22°C. to 30.6°C. is proportional to the increase in volatility over the same temperature range. Thus when the penetration rate for H at 21-22°C. is divided by the volatility at this temperature, the quotient is about

1.9. A similar quotient (1.85) results from the data obtained at 30.6°C. The same considerations hold for EBA, the quotients for the two temperatures being slightly lower, namely, 1.5 to 1.6. For TBA, however, the quotients for the two temperatures do not agree so well. The reason for the divergence in this case cannot be stated with assurance. It should be pointed out, however, that there is little agreement between the values reported in the literature for the volatility of TBA. The low volatility and the low penetration rate of TBA make the accurate determination of either value a matter of some difficulty.

It should be noted that all of the quotients given in Table XII are in the same range. Thus it would appear that, for the temperatures investigated, the penetration rates of H, EBA, and TBA are nearly proportional to the respective

TABLE XII  
*Correlation of Penetration Rate and Volatility*

Agent	Temperature	Volatility*	Penetration rate
			Volatility
H	°C.	mg. per l.	
	21-22	0.71-0.77‡	2.0-1.8
EBA	30.6	1.46‡	1.85
	22	1.73-1.87‡	1.6-1.5
TBA	30-31	3.24-3.39‡	1.6-1.5
	22-23	0.094-0.102‡	1.9-1.8
	30	0.181‡	1.6

\* The exact temperature inside the penetration cup during the exposure has not been determined. Hence the volatilities of the agents are calculated on the assumption that the temperature of the liquid vesicant inside the cup is the same as that of the room.

‡ Calculated from data in (6).

volatilities, and are not markedly dependent upon the differences in chemical structure of the agents. This generalization, which appears to be valid to within about 15 per cent, suggests that volatility is the principal factor governing the penetration rates of the three agents studied. From these experiments it would appear, therefore, that at the same gas concentration in milligrams per liter H, EBA, and TBA would penetrate the human skin at the same rate. Consequently, differences in vesicancy of these agents (and benzyl-H) cannot be attributed to variation in their ability to penetrate human skin.

#### 6. The Effect of Temperature on the Vesicant Action of H, EBA, and TBA Vapors

The data presented in section 4 of this communication permit one to determine whether, under our experimental conditions, the increase in temperature

from 22°C. to 30°C. causes any appreciable change in the vesicancy of the three agents under study. In Table XIII, the results have been arranged so as to show the amount (in gammas per cm.<sup>2</sup>) of each vesicant which must penetrate to cause vesication in approximately 50 per cent of the exposed sites. This amount, which was determined by inspection of the data, will be designated by the symbol  $V_{50}$ . As was mentioned earlier, there has not been an opportunity to ascertain whether any of the vesicant vapor adsorbed by the skin is lost as a result of postexposure evaporation. In the calculation of the  $V_{50}$  doses, presented in Table XIII, it has been assumed that all of the vesicant which disappears from the penetration cup during exposure has been retained by the skin.

It will be noted from Table XIII that H and TBA are approximately equally effective per gamma penetrated, not only at 21–23°C. but also at 30–31°C. Furthermore, no striking difference in  $V_{50}$  was noted for either agent when the temperature was raised. Similarly, experiments with EBA did not show any differences in effectiveness at the two temperatures. From these data it would appear also that EBA is a relatively poor vesicant at either temperature, being only  $\frac{1}{2}$  to  $\frac{1}{3}$  as effective per gamma penetrated as is H or TBA.

It may be of interest to call attention to the small difference between the dose of vesicant necessary to produce erythema, and that necessary to produce vesication. This is particularly apparent in the case of H and TBA vapor at 22°C. With H vapor after 4  $\gamma$  had penetrated, no vesicles appeared at twelve sites. After 8  $\gamma$  had penetrated, however, seven of nine sites vesicated. With TBA vapor, after 5  $\gamma$  had penetrated, only one of eleven sites vesicated, while after 8  $\gamma$  had penetrated, seven of nine sites vesicated.

From the investigations reported above, it would appear that, under the conditions prevailing in these experiments, the skin of the human forearm is not markedly more sensitive at 30°C. to the vesicant agents than it is at 22°C. Since this conclusion is at variance with other findings reported in the literature (7), the conditions under which these experiments were performed should be considered in some detail.

In the first place it should be mentioned that the subjects employed in our experiments were for the most part Chemical Warfare Service personnel, and about 80 per cent of them had previously been exposed to and burned by vesicant agents. Hence it is not impossible that many of these men had become sensitized to one or more of the agents studied. In a few cases definite evidence of hypersensitivity was manifest (excessive local damage, flaring up of old lesions in remote areas, etc.). The vast majority of the subjects appeared to give normal responses, and sensitivity was suspected only on the basis of the histories. The hypersensitive subjects exhibited no significant differences in penetration rates compared to the other subjects, however, and it seems unlikely that hypersensitivity would be associated with an increased penetration rate.

On the other hand, the physiological response of hypersensitive subjects would be abnormal. Thus the validity of any conclusions based upon the physiologi-

TABLE XIII  
*The Effect of Temperature on the Vesicant Action of H, EBA, and TBA Vapors*

Agent	Tempera-ture	Ct*(ap-proximate)	Amount of agent pene-trated	No. of men	No. of sites	No. of lesions 48 hrs. after exposure				Approximate $V_{50}$ γ/cm. <sup>2</sup>
						O	E	PV†	V	
	°C.	mg. min./ m <sup>2</sup> .	γ/cm. <sup>2</sup>							
H	21-23	2300	4	6	12	0	12	0	0	6
		4600	8	6	9	0	2	0	7	
		7700	14	7	11	0	0	0	11	
	30.6	2900	5	12	24	0	10	2	12	5
		5800	11	12	18	0	0	0	18	
		8800	16	6	6	0	0	0	6	
EBA	22	9000	14	6	12	3	7	2	0	28
		18000	28	6	12	0	4	4	4	
		27000	42	6	12	0	1	9	2	
		36000	56	5	10	0	1	3	6	
	30-31§	8000	13	9	12	4	6	0	2	26
		16500	26	9	15	0	8	0	7	
		24500	39	9	9	0	1	0	8	
TBA	22-23	2000	4	3	3	0	3	0	0	6.5
		2900	5	6	11	0	10	0	1	
		4400	8	6	9	0	2	0	7	
		5900	11	6	12	0	2	0	10	
	30	2700	4	9	15	0	7	2	6	4
		5400	9	6	12	0	0	0	12	
		8000	13	6	9	0	0	0	9	

\* These values are calculated upon the assumption that the temperature inside the cup is the same as that of the room and that the air inside the cup is saturated with respect to vesicant.

† In evaluating the  $V_{50}$  doses, pinhead vesicles (PV) were arbitrarily selected as representing a state midway between erythema (E) and vesication (V).

§ The clinical observations on the Negro subjects are not included in this summary.

cal response of the subjects depends upon the extent to which the group investigated deviates from the normal.

It is a necessary consequence of the technique employed in these experiments that the air inside the penetration cup undoubtedly possessed a very high relative humidity. In the experiments conducted at 30°C. the atmosphere inside the cup probably became saturated with water vapor in a relatively short time. At 22°C. the time necessary to achieve saturation was undoubtedly longer.

However, in both experiments it seems possible that the moisture content of the skin was about the same. These considerations are of great importance in view of the results of Smith *et al.* (4) and Renshaw (8) which indicate that the presence of excess moisture on the skin surface has a marked effect upon the damage produced by a given concentration of vesicant vapor.

In evaluating local damage produced by a given dose of vesicant, it seems possible that the severity of the lesion may be influenced not only by the site, but also by the size of the area exposed. The  $V_{50}$  doses given in Table XIII, therefore, may only be applicable to a lesion which is located on the forearm and has an area of about 1.3 cm<sup>2</sup>.

The physiological response of the subjects might also be a function of the season of the year during which the exposures took place. The  $V_{50}$  doses for H at 21–22°C., and for H, EBA, and TBA at 30–31°C. were determined in New York City during late October and early November. The  $V_{50}$  dose for TBA at 22–23°C. was determined in part at the same season, and in part during late August. The  $V_{50}$  dose for EBA at 22°C. was determined during late September.

We wish to acknowledge gratefully the cooperation of the volunteers in the conduct of the experiments, and should like also to thank Colonel C. P. Rhoads, Lieutenant Colonel A. M. Bowes, Major W. H. Sherwin, Dr. H. W. Smith, and Commander M. B. Sulzberger for their valuable assistance in securing these volunteers. In the course of the experiments, we enjoyed the helpful cooperation of Commander M. B. Sulzberger and Dr. R. L. Baer in the clinical evaluation of the lesions. We are also grateful to them for many profitable discussions concerning the results.

#### SUMMARY

Analytical methods which are accurate to about 1 per cent have been developed for the determination of small amounts (*ca.* 500 γ) of bis(β-chloroethyl)sulfide (H), ethyl-bis(β-chloroethyl)amine (EBA), tris(β-chloroethyl)amine (TBA), β-chloroethyl-benzylsulfide (benzyl-H), and β-chloroethyl-ethylsulfide (ethyl-H). The determinations are made by micro titration of the HCl liberated upon complete hydrolysis of the vesicants.

A description is given of an apparatus suitable for applying vapors of vesicants to unit areas of skin. A very precise and reproducible micropipetting technique is described for the introduction of the vesicants into the penetration apparatus.

By means of this penetration apparatus studies have been made of several factors which may influence the rate at which vesicant vapors penetrate into skin. Model experiments have been performed in which H was allowed to vaporize and the vapor was absorbed on a surface such as that of diethylene glycol or vaseline. It has been found that if the surface of liquid H is increased by spreading the agent on filter paper, the rate of evaporation is markedly increased. Furthermore, if the vapor is agitated by means of a magnetically

driven fan, the rate of absorption by diethylene glycol is greatly accelerated. With vaseline as the absorbing surface it has been found that the area of the absorbing surface has an effect on the rate of absorption of H vapor. More H is absorbed by vaseline spread on filter paper to give a rough surface than is absorbed by a smooth film of vaseline.

Measurements of the rate of penetration into human skin of H, EBA, TBA, benzyl-H, and ethyl-H vapors have been performed at 21-23°C. and 30-31°C. by means of the penetration apparatus described in this paper. The measurements were carried out on human volunteers under conditions of controlled temperature and humidity.

When human skin is exposed to air saturated with H vapor, the H penetrates the skin of the forearm at a rate of about 1.4 γ per cm.<sup>2</sup> per minute (temperature 21-23°C.; relative humidity 46 per cent). This value was found to hold in experiments in which H vapor was applied for 3 to 30 minute intervals, thus indicating that the permeability of the skin to H vapor is not altered during a 30 minute exposure. Agitation of the H vapor by fanning did not result in any measurable increase in the rate of penetration. Two of the volunteers were Negroes; the permeability of their skin to H vapor did not differ appreciably from that found for the other subjects. When human skin is exposed to air saturated with EBA vapor, the vesicant penetrates at the rate of 2.8 γ per cm.<sup>2</sup> per minute (temperature 22°C., relative humidity 50 to 52 per cent). The amount of EBA penetrated is linear with exposure time for exposure periods of 5 to 20 minutes. Under similar conditions, it was found that TBA penetrates at a rate of about 0.18 γ per cm.<sup>2</sup> per minute (temperature 22-23°C.; relative humidity 45 to 48 per cent). This value was found to hold in experiments in which TBA vapor was applied for 30 to 60 minute intervals. The amount of TBA penetrated is linear with exposure time. In the case of benzyl-H, a linear relationship between the amount lost from the penetration cup and exposure time was also observed but the plot did not pass through the origin. It is suggested that this anomaly is due to retention on the skin surface of an appreciable quantity of benzyl-H as a result of rapid physical adsorption or chemical combination with a constituent of the skin. The rate of penetration of benzyl-H may be calculated from the slope of the plot and is found to be 0.35 γ per cm.<sup>2</sup> per minute (temperature 22°C., relative humidity 55 to 60 per cent). The results with ethyl-H showed great variation among individual subjects and no satisfactory value for the rate of penetration can be given as yet.

Measurements were also made of the rate of penetration of H, EBA, and TBA vapors at 30-31°C. (relative humidity 47 to 49 per cent). At this temperature, a linear relationship was observed between the amount penetrated and the time of exposure. H vapor penetrated at a rate of 2.7 γ per cm.<sup>2</sup> per minute, EBA vapor at 5.1 γ per cm.<sup>2</sup> per minute, and TBA vapor at 0.29 γ per cm.<sup>2</sup> per minute. Three of the subjects in the EBA measurements were Negroes. The

permeability of their skin to EBA vapor did not differ from that found for the white subjects. Despite this fact, their skin failed to vesicate after an exposure period twice that which caused 50 per cent vesication in the white subjects.

Calculation of the precision of the data showed that the average deviation, standard deviation, and standard error were not appreciably different for the data obtained with human subjects as compared with data for control experiments in which human skin was not involved. Consequently, no significant differences in the rate of penetration into the skin of individual subjects can be discerned from the data presented in this communication.

The increase in the rate of penetration of H, EBA, and TBA vapors from 21-23°C. to 30-31°C. is approximately proportional to the increase in volatility of each agent. These results indicate that at the same gas concentration in milligrams per liter, H, EBA, and TBA vapors would all penetrate at about the same rate.

The data presented above permit a determination of the approximate amount of each vesicant which must penetrate to cause vesication in about 50 per cent of the exposed sites. This amount has been designated by the symbol  $V_{50}$ . The  $V_{50}$  for H and TBA at 21-23°C. is the same, being about  $6\gamma$ ; at 30-31°C., the  $V_{50}$  is 4 to 5  $\gamma$ . On the other hand, the  $V_{50}$  for EBA at 22°C. and 30-31°C. is about 26 to 28  $\gamma$ . Thus, per gamma penetrated, H and TBA vapors are about equally effective in producing vesication while EBA vapor is only  $\frac{1}{3}$  to  $\frac{1}{2}$  as effective.

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